The Dissertation Committee for Megan Elizabeth Tipps certifies that this is the approved version of the following dissertation:

Identification of Novel Allosteric Modulators of the Glycine Receptor Using Phage Display Technology

Committee:

_____
S. John Mihic, Supervisor

_____
Richard W. Aldrich

_____
R. Adron Harris

_____
Brent L. Iverson

_____
Harold H. Zakon

Identification of Novel Allosteric Modulators of the Glycine Receptor Using Phage Display Technology

by

Megan Elizabeth Tipps, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

University of Texas at Austin

August 2011

Dedication

This dissertation is dedicated to my parents, David and Elizabeth, who gave up their own paths to ensure that I could choose mine.

Acknowledgements

This dissertation is the product of hard work, long hours, and many helping hands. It is impossible for me to thank every person that contributed, but I would like to take a moment and thank those who have had the largest impact on my success in graduate school.

First, I would like to thank my mentor, Dr. S. John Mihic, for his constant support and kindness. His willingness to work through problems and give even my most far-fetched ideas a shot has taught me to be fearless with my work and not take unexpected results too personally. From the first day I walked into his lab, he has radiated an enthusiasm about this work and science in general that has helped me push through even the most daunting roadblocks. He has been present for many major changes in my life, and he has taken each of them in stride and supported me without question. He is largely responsible for the scientist I am today, and I can only hope that turns out to be a compliment.

I would also like to thank the members of my dissertation committee. Dr. Richard Aldrich, Chair of the Neurobiology department, is the source of my deep affection for ion channels. His wonderful sense of humor and seemingly boundless knowledge (of just about everything) has made working with him a truly wonderful experience. Dr. Adron Harris, Director of the Waggoner Center for Alcohol Addiction Research, has always made time to discuss the various facets of my work and often kept me company during weekend experiments. He has taught me to constantly look forward, both in my own

work and in the addiction field as a whole, helping prepare me to become a productive member of the research community. Dr. Brent Iverson, Chair of the Chemistry department, has been so very kind and patient throughout the development of this project. His willingness to take the time to explain even the most basic biochemical principles made this project possible. Dr. Harold Zakon was one of the first professors I met upon joining the Neuroscience program. He has always been ready with a kind word and a completely unique perspective on my work, two things that have been absolutely invaluable to me. Even the most dedicated scientist can go astray without guidance. The members of my committee were always there to steer me in the right direction, and for that I am eternally grateful.

I have been lucky to work with several other excellent scientists along the way. Dr. Michelle Dupre Bayly took on the arduous task of training me when I joined the lab and along the way become a wonderful friend. Dr. Luisa Scott was foolish enough to let me talk her into attempting phage display with me. Her determination and wealth of knowledge were all that held this project together on some days. Dr. Sangeetha Iyer, to whom I leave my phage legacy, has approached this project with great enthusiasm and energy. I could not ask for a better scientist or coffee partner to share my work with. Dr. Jelena Todorovic, Dr. Brian Welsh, and Dean Kirson have all been excellent labmates. They patiently sat through many phage discussions and were always full of helpful suggestions. I have also had the honor of mentoring three undergraduate students in my time here. Jessica Lawshe, Hunter Allen, and Joshua Klaus have been enthusiastic and

dedicated workers. I have enjoyed getting to know each of them, and I know they will go on to do great things.

I would also like to thank Krystal Phu, the graduate coordinator for the Institute of Neuroscience for ensuring that I never missed a deadline. As part of the Waggoner Center, Debbie James and Marsha Berkman have provided me with exceptional administrative (and often emotional) support. I am convinced that there is not a problem in the world that cannot be solved by these three women.

I was fortunate to receive several sources of financial support, including an institutional training grant through the University of Texas Neuroscience Graduate Program and the NIAAA training grant run by Dr. Ruben Gonzalez. I was also supported by my NIAAA grant and the Bruce/Jones Predoctoral Fellowship.

In the non-science sector, I would like to thank my partner, Jaime Jones, for holding my hand and constantly encouraging me every step of the way. There were several days over the course of this project in which I was certain that all of this was impossible, but she never waivered in her faith in me. She has been my best person for over a decade now, and I could not have asked for a more loving and supportive person to share my life with. Finally, thanks to my family. My parents have always been there to support me, and without their love and dedication I would not be who I am today. My sisters, Hillary and Kelsey, have no idea what it is I do, but they love me anyway, as I do them. We are an odd clan, but we like it that way.

There are many other friends and co-workers that have contributed to this project, either directly through their scientific suggestions or by keeping me calm and sane. It has been an honor to know and work with you all.

Identification of Novel Allosteric Modulators of the Glycine Receptor Using Phage Display Technology

Publication No._____

Megan Elizabeth Tipps, Ph.D.

The University of Texas at Austin, 2011

Supervisor: S. John Mihic

The glycine receptor (GlyR) is a ligand-gated ion channel and a member of the cys-loop receptor family. Like other members of this family, the GlyR is a target for many drugs of abuse, including alcohol. While the effects of alcohol on these receptors have been well-characterized, the contribution of each receptor subtype to the overall physiological and behavioral effects of alcohol use are unclear. This is partially due to the limited pharmacology of the GlyR, which limits the ability to isolate GlyR function within a complex system. One method for identifying compounds that bind to and modulate a given target is phage display. This approach uses bacteriophage to screen a

large number of peptide sequences for affinity at a given target. We developed a phage selection protocol to identify peptides that bind to the GlyR. These peptides were then tested for functional effects at the GlyR using two-electrode voltage clamp physiology. We identified several peptides that were able to modulate GlyR function. Peptide D12-116 showed specificity for the GlyR over two closely related γ-aminobutyric acid (GABA) channels. In addition, this method is easily adapted for the selection of peptides that bind to any cell-expressed target, increasing the utility of phage display in the neurobiology field. Another shortcoming in GlyR pharmacology is the lack of modulators with specificity for a single GlyR subtype. We next adjusted our selection protocol to search for peptides that can distinguish between the different Gly R α subtypes. We identified several promising lead peptides that show subtype preference. Finally, we found that trifluoroacetic acid (TFA), a common peptide contaminant, also modulates GlyR function. This finding has important implications for both previously reported peptide modulators and the pharmacology of several volatile anesthetics, for which TFA is the major metabolite.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5-HT_3R	serotonin 3 receptor
BSA	bovine serum albumin
CHO	Chinese hamster ovarian
CMV	cytomegalovirus
CNS	central nervous system
CPP	cell penetrating peptide
EC_{10}	Effective concentration (10%)
ECD	extracellular domain
ELIC	*Escherichia coli* ligand-gated ion channel
GABA	γ-aminobutyric acid
GLIC	*Gleobacter violaceus* ligand-gated ion channel
GluCl	glutamate-gated chloride channel
GlyR	glycine receptor
HCFC	hydrochlorfluorocarbons
HEK	human embryonic kidney
HFC	hydrofluorocarbons
HPLC	high-performance liquid chromatography
ICD	intracellular domain
K_{ATP}	ATP-gated potassium
LGIC	ligand gated ion channel
LORR	loss of righting reflex

MBS	modified Barth's solution
NAcc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
PBS	phosphate-buffered saline
PD	phage display
PEG	polyethylene glycol
PKA	protein kinase A
PKC	protein kinase C
PMTS	propyl methanethiosulfonate
PTX	picrotoxin
RF	replicative form
TFA	trifluoroacetate or trifluoroacetic acid
T_m	melting temperature
TM	transmembrane
VA	volatile anesthetics
VTA	ventral tegmental area
Zn^{2+}	zinc

Amino acid residue abbreviations and naming conventions

Amino acids residues are typically abbreviated using single letters, as in the list below. Point mutations within a protein's structure are described using the following nomenclature: (old residue)(residue position)(new residue). For example, if the serine at

position 267 is mutated into a cysteine residue, the mutant protein is referred to as S267C.

Single-letter amino acid abbreviations

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartate	P	Proline
E	Glutamate	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

1.0 | INTRODUCTION

A multitude of compounds alter the function of the central nervous system (CNS) by modulating various enzymes, transporters, and receptors. Some compounds, like benzodiazepines, act with high specificity at a single target (Mihic et al., 1994). However, many of these compounds alter the function of several targets simultaneously, resulting in a complex mechanism of action. This complexity makes it difficult to assess which of the many biological targets of the compound are important for which aspects of the behavioral effects elicited. One approach for addressing this question is the development of compounds that mimic the effect of the compound or specifically block the effect of the compound at only one of its biological targets. These highly specific modulators could then be used to determine how changes at individual targets contribute to the complex behavioral response elicited by the original compound. In this thesis, I explore one of the methods for identifying novel modulators, phage display, and use it to identify modulators for a specific target of a widely used CNS drug, alcohol.

1.1 – Alcohol

Alcohol, the common term for ethanol, is one of the most commonly used drugs of abuse. With the exception of nicotine addition, alcoholism is the most common addictive disorder, due primarily to its high accessibility and the social acceptability of its use. In addition to the health risks associated with alcohol use, such as cirrhosis of the

liver and heart disease, ethanol misuse has wider social implications. According to the center for disease control, alcohol is involved in two out of three violent crimes, and causes an estimated 75,000 deaths annually. In spite of centuries of ethanol use and the significant social impact of this drug, the mechanism by which ethanol exerts its actions in the central nervous system (CNS) remain poorly understood.

The original mechanism of action proposed for ethanol involved non-specific interactions with the constituents of cell membranes, in particular lipids. This theory was based on the observation by Meyer and Overton that the potencies of alcohols and general anesthetics were proportional to their partition coefficients between oil and water (Franks and Lieb, 1997a). Short chain alcohols are amphiphilic, and thus localize primarily in the head group region of the lipid bilayer. The inclusion of alcohols in the cell membrane disrupts the packing of the lipid components, leading to an increase in fluidity, permeability, and lateral mobility (Frischknecht and Frink, 2006). Chronic ethanol administration causes biophysical changes in membranes that result in resistance to this lipid-disordering effect (Goldstein and Chin, 1981), and these changes coincide with the development of behavioral tolerance.

However, several aspects of alcohol behavior are inconsistent with this theory. First, the degree of lipid disorganization seen at intoxicating concentrations of ethanol is very small and is also seen when the temperature is raised by only $1^{o}C$. This temperature change has no effect on behavior, suggesting that lipid disorganization alone is not sufficient to induce intoxication (Franks, 2006). In agreement with this, A_2C, a compound that produces membrane fluidization and mobility similar to that of anesthetic

agents, fails to produce anesthetic-like effects (Buck et al., 1989). The lipid theory is also unable to account for the cut-off effect seen in alcohols. Increasing the carbon backbone chain length of alcohols increases their membrane:buffer partition coefficients; however, this is not always correlated with an increase in alcohol potency, and a cut-off is observed at which further increases in alcohol potency are not seen, despite increasing chain length (Pringle et al., 1981). In addition, several anesthetic compounds have optical isomers that, despite being equally lipid soluble, have significantly different anesthetic potencies (Franks and Lieb, 1997b). The most important shortcoming of this theory is its inability to explain how nonspecific changes in the membrane eventually alter the function of membrane-associated proteins, even though this is widely accepted to be the ultimate result (Franks, 2006). These inconsistencies led to a search for alternate sights of action for alcohols and anesthetics. In the early 1980s, Franks and Lieb (1984) demonstrated that clinically relevant concentrations of anesthetics could inhibit the function of the cytosolic enzyme firefly luciferase. As this enzyme is not embedded in the membrane, nonspecific membrane effects do not alter its function. The anesthetic inhibition of luciferase function not only showed good correlation with the concentrations used to achieve anesthesia in animals, but also demonstrated a cut-off effect, suggesting that the anesthetics were interacting with the enzyme at a binding site with fixed dimensions (Franks and Lieb, 1985). This discovery instigated the search for CNS proteins that were directly modulated by alcohols and anesthetics at behaviorally relevant concentrations. An abundance of electrophysiological and biochemical evidence for the modulation of ion channels by alcohols and anesthetics had been reported over the years (Cheng and

Brunner, 1985; Huidobro-Toro et al., 1987; Gage and Robertson, 1985; Buck et al., 1989); however, it was unclear if these effects were due to direct interactions with channel proteins or indirect effects on lipids. Conclusive evidence for direct alcohol-protein interactions was provided by the crystallization of LUSH, an odorant binding protein found in *Drosophila melanogaster* (Kim et al., 1998). The LUSH protein was crystalized in the presence of various alcohols, and the structure showed an ethanol molecule stabilized by hydrogen bonds at a binding site with a threonine-serine-threonine motif. This provided direct evidence for alcohols binding at a discrete site on a protein (Kruse et al., 2003).

The evidence for proteins as direct targets for alcohol, such as luciferase, initiated the search for alcohol binding sites on CNS proteins. Motifs similar to the one identified in LUSH can be found in several CNS proteins whose functions are modulated by alcohols, and mutational studies have identified specific residues on several CNS enzymes and channels that mediate the effects of ethanol. As a full review of this work would be quite extensive, the identification of alcohol binding sites will be limited to only a few targets and will be discussed in section **1.3.3**.

In the past three decades, a large number of putative ethanol targets have been identified, including various enzymes and ion channels. While the identification of possible protein targets for ethanol has been highly successful, the large number of targets complicates the efforts to understand the specific mechanism of action for this drug. In addition, most of these targets only show significant modulation in response to higher concentrations of ethanol (50 – 200 mM), while behavioral intoxication occurs at

much lower concentrations (5 – 20 mM). This suggests that the effects of low concentrations of ethanol are either mediated by as of yet unidentified targets, or through a combination of small changes in several systems. Thus, one of the central questions in the alcohol research field today is how the modulations of a large number of putative protein targets combine to generate the complex behavioral responses seen in response to ethanol.

1.2 - Cys-loop Family of Receptors

Ion channels form the basis of the inter-neuronal communication in the neural system, allowing neurons in the CNS to communicate through chemical and electrical signals. As previously mentioned, the function of several families of ion channels is modulated by ethanol. Of these channels, the Cys-loop family of receptors has been extensively studied with regards to ethanol effects. This family consists of two anion-conduction channels, γ-aminobutyric acid type A and C ($GABA_{A/C}$) and glycine receptors (GlyR), and three cation-conduction channels, serotonin type 3 ($5\text{-}HT_3$), nicotinic acetylcholine (nACh), and the recently discovered zinc-activated channels (Davies et al., 2003; Lynch, 2004; Lester et al., 2004). Ethanol affects almost all of the members of this family, and these modulatory effects are consistent with several of the behavioral effects of ethanol intoxication (Hyytia and Koob, 1995; Narahashi et al., 1999; Lovinger, 1997). For the purposes of this thesis, I will focus my discussion on a single member of this family, the glycine receptor (GlyR). However, I will start with a brief overview of general cys-loop receptor structure and function.

1.2.1 – Cys-loop Receptor Structure

The members of this receptor family are membrane-spanning ion channels that are activated by neurotransmitters (ligand-gated) and are responsible for fast excitatory and inhibitory transmission. Agonist binding leads to the opening of an integral ion-selective channel that allows the passive transport of permeant ions, resulting in either depolarization or hyperpolarization of the neuronal membrane (Maksay, 2009). Cys-loop channels have several common characteristics that have been largely elucidated from the electron microscopic structure of the *Torpedo* nicotinic acetylcholine receptor (nAChR) and the crystal structure of the ACh binding protein (Brejc et al., 2001; Unwin, 1995). The more recent crystallization of several eukaryotic channels (GLIC, ELIC, and GluCl) has further enhanced our understanding of how the structure and function of these channels are related (Hilf and Dutzler, 2008; Bocquet et al., 2009; Hibbs and Gouaux, 2011).

The basic receptor structure is formed by five subunits distributed around a central ion-conducting pore. Each of the five subunits contains four transmembrane (TM) domains, with the second domain (TM2) contributing to the formation of the pore (Figure 1.1). The large extracellular N-terminal domain (ECD) contains both the neurotransmitter binding site and the characteristic cysteine disulfide bonds that give this family of channels their cys-loop structure. A large intracellular domain (ICD), consisting primarily of a loop of amino acids connecting TM3 and TM4, is involved in channel sorting, trafficking and modulation by intracellular factors (Maksay, 2009).

These components will be discussed in detail as they pertain to the GlyR in the next section.

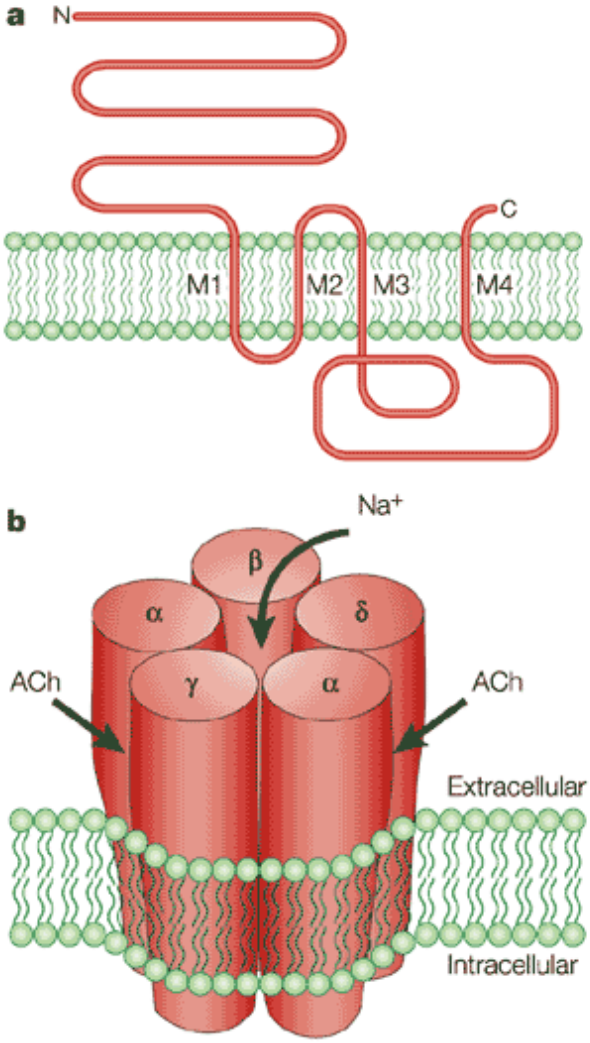

Figure 1.1 – Schematic of a nicotinic acetylcholine receptor

(A) Illustration of a nicotinic acetylcholine receptor subunit showing the extracellular N-terminal ligand binding domain, the four transmembrane domains (M1 – M4), and the intracellular loop connecting M3 and M4. *(B)* Five subunits are arranged to form a single receptor. The intersubunit acetylcholine binding sites and the central ion-conducting pore are indicated. *Adapted from Karlin A. (2002). Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci **3:**103.*

1.3 - Glycine Receptor

Glycine was first proposed to be a neurotransmitter in the spinal cord, where it has an inhibitory effect on motoneuron excitability (Davidoff et al., 1967; Werman et al., 1967). Glycine, synthesized from serine by serine-hydroxymethyltransferase, is packaged into synaptic vesicles and released following an action potential cascade (Daly and Aprison, 1974; Legendre, 2001). The first attempts to clone glycine-activated channels yielded two subunits, the 48 kDa α1 subunit and the 58 kDa β subunit (Pfeiffer et al., 1982). Based on the homology of the a1 subunit with the previously cloned subunits of the nAChR, the GlyR was classified as a member of the Cys-loop family of receptors (Grenningloh et al., 1987b).

Subsequent homology screens identified two more α subunits (α2 and α3). A fourth a subunit (α4) was identified in mice and chicks, but is only a pseudo-gene in humans (Lynch, 2004). These subunits form pentamers consisting of either five a subunits (homomeric receptors) or a mixture of α and β subunits (heteromeric receptors). The inclusion of the β subunit causes a significant reduction in single channel conductance and changes in channel kinetic behavior compared to homomeric receptors (Beato et al., 2004). While the β subunit cannot form homomeric channels (Bormann et al., 1993), it is necessary for interactions with various intracellular components, most notably gephryn, which mediates receptor clustering at the synapse (Fritschy et al., 2008). Based on electrophysiological measurements, the stoichiometry of heteromeric GlyRs was originally thought to be 3 α and 2 β subunits; however, more recent studies using

tandem constructs suggest that these channels actually consist of 2 α and 3 β subunits (Langosch et al., 1988; Grudzinska et al., 2005).

1.3.1 – Activation and Gating of the GlyR

As with other cys-loop receptors, the GlyR is designed to mediate fast neural transmission. In the absence of neurotransmitter, these channels exist in a closed state with extremely low or non-existent spontaneous opening events. In addition to glycine, these channels can also be activated by other amino acids, most notably β-alanine and taurine. Glycine is more efficacious than the other ligands (Lynch et al., 1997), which are typically thought to act as partial agonists on these channels. However, the levels of taurine in some brain regions have led to speculation that it may act as the primary ligand of GlyR in certain areas (Mori et al., 2002; Ericson et al., 2006).

During channel activation, glycine binds to the ligand-binding site in the ECD. The binding site is a pocket-like structure formed by b-sheets connected by flexible loops (Brejc et al., 2001). This site is located between subunits, with three loops from one subunit forming the principal binding site (domains A-C) and three β-sheets from an adjacent subunit contributing a complimentary binding site (domains E-F). Thus, each homomeric GlyR is thought to have a total of five possible ligand-binding sites. However, the binding of only three glycine molecules is sufficient to fully activate these channels (Beato et al., 2004). Based on work done using the ACh binding protein (AChBP) structure, glycine is hypothesized to be stabilized within the binding site by interactions with aromatic residues, primarily by π-cation interactions (Zacharias and

Dougherty, 2002). Consistent with this theory, mutations that removed aromatic residues from the hypothesized binding pocket altered the sensitivity of GlyR to glycine, taurine, and β-alanine.

When a neurotransmitter binds to a cys-loop receptor, a cascade of movement within the channel protein, described as a Brownian conformational wave, carries the binding signal to the gate (Grosman et al., 2000). Unwin (1995) proposed that agonist binding initiated a rotation in the binding region that enables contact between the β-sheets of the ECD and the extracellular linker region that connects TM2 with TM3. The movement of this linker region then causes the TM2 segments lining the pore to twist away from one another, allowing ions to flow down their concentration gradients (Miyazawa et al., 2003). A ring of positively charged residues within the pore serves as a selectivity filter allowing only anions, primarily chloride, to flow through. The role of the remaining two TM segments in channel activation and gating is less clear. However, this region is involved in the modulation of these channels by several second messenger systems, including PKC, PKA, PKT, (Lynch, 2004) and Gbg (Yevenes et al., 2003). In addition, mutations in this region have been shown to partially account for the extremely reduced taurine responses in α3-containing receptors compared to a1-containing receptors, suggesting a possible role for this region in the modulation of the binding signal (Chen et al., 2009). The pore closes when either the ligand dissociates away from the receptor or the receptor enters a desensitized state (Lynch, 2004).

1.3.2 – Localization and Function of the GlyR

Original localization experiments using [^{3}H]strychnine, a highly specific GlyR competitive antagonist, found high levels of surface-expressed GlyRs in the spinal cord, pons, thalamus, and hypothalamus (Zarbin et al., 1981). Later work with a GlyR α-subunit antibody reported additional distributions of GlyR in the cerebellum, olfactory bulb, and hippocampus (van den Pol and Gorcs, 1988). GlyRs have also been found in several structures of the basal ganglia circuit, a region that is important for motor control and mood (Waldvogel et al., 2007). These receptors are abundantly expressed in the retina, where they are thought to play a role in the transition from day to night vision (Grunert, 2000; Ivanova et al., 2006). Recently, GlyR expression has also been reported in forebrain regions. The expression of GlyRs in the basolateral amygdala suggests that these receptors might play a role in anxiety disorders (McCool and Botting, 2000). Of particular relevance to this thesis, GlyRs were recently found in the nucleus accumbens (NAcc) and the ventral tegmental area (VTA), regions that play important roles in the reinforcing effects of drugs of abuse (Jonsson et al., 2009; Ye et al., 2001a). This discovery led to a renewed interest in the GlyR as a modulator of ethanol-related behaviors.

The abundance of these receptors in the spinal cord makes them the dominant inhibitory system in the brainstem and spinal cord. The influx of chloride ions through the GlyR channel has an inhibitory effect on neuronal excitability, thus mutations or deletions of this channel lead to startle phenotypes (Lewis et al., 1998; Lynch et al., 1997). In humans, disruptions in GlyR function typically manifest as hyperekplexia, a

disorder characterized by an exaggerated response to unexpected stimuli that is often accompanied by temporary muscular rigidity (Rajendra and Schofield, 1995). This disorder is the result of a reduction the magnitude of glycine-gated chloride currents, and several mutations throughout the channel have been linked to this disease (Lynch, 2004).

Traditionally, the α1 subunit was thought to be the most abundant subunit in the adult CNS, and thus the majority of GlyR research focused exclusively on this subtype. However, the advent of improved localization techniques and subunit-specific antibodies have allowed for a more detailed analysis of GlyR subunit expression (Malosio et al., 1991). For example, in the adult hippocampus, extrasynaptic α2 and α3 subtypes and somatic α3-containing receptors are more abundant than α1/α1β receptors (Aroeira et al., 2011). GlyR expression in the amygdala varies by region, with α1β expression dominating the basolateral amygdala and α2β and α3β expressed highly in the central region (Delaney et al., 2010). All four subunits (α1-3 and β) are expressed in the NAcc, but α2 is the most prevalent (Jonsson et al., 2009). Although the various a subunits are highly homologous in amino acid sequence, these channels differ in their sensitivities to ligands and several modulators (Lynch, 2009), suggesting that the differential expression of these subunits could serve as a fine-tuning mechanism in GlyR transmission. For example, in the spinal cord, α1β channels are the dominant form, but only the α3-containing receptors are thought to contribute to the GlyR modulation of inflammatory pain (Zeilhofer, 2005).

1.3.3 - GlyR and Alcohol

The enhancing effect of ethanol on glycine-mediated currents was first demonstrated in chick spinal neurons (Celentano et al., 1988). This potentiation was subsequently confirmed in brain synaptosomes, isolated neurons, and human GlyRs expressed in heterologous systems (Engblom and Akerman, 1991; Aguayo and Pancetti, 1994; Eggers et al., 2000; Mascia et al., 1996a). Pharmacologically relevant concentrations (50-100 mM) of ethanol cause a persistent, reversible increase in glycine-mediated currents. This change is not caused by increases in either presynaptic events or GlyR single channel conductance levels, suggesting that ethanol increases the probability of GlyR channel opening (Eggers et al., 2000).

Based on the cutoff effect seen with long-chain alcohols, ethanol was hypothesized to bind at a specific site with discrete dimensions (Mascia et al., 1996a). In an attempt to locate this binding site, Mihic et al. (1997) generated chimeric channels using segments from the GlyR and $GABA_C$ channels. Unlike other members of the $GABA_{A/C}$ family, $GABA_C$ receptors are inhibited by ethanol (Mihic and Harris, 1996). By combining segments from GlyR and $GABA_C$ subunits, the authors were able to identify the region between TM2 and TM3 as the area responsible for mediating the effects of ethanol and the volatile anesthetic isoflurane. Two residues within this region, S267 and A288, were shown to be critical for the potentiating effects of ethanol on the GlyR (Mihic et al., 1997). The size of the residue at the S267 position is inversely correlated with the potentiating effects of ethanol (Ye et al., 1998), suggesting that this residue contributes to the formation of a “binding pocket” of set dimensions. Cysteine

accessibility experiments, in which individual residues are mutated to cysteines and tested for their abilities to crosslink with various thiol reagents, have been used to investigate whether binding to these residues is sufficient to modulate channel activity. The covalent binding of either propanethiol or propyl methanethiosulfonate (PMTS) to the S267C leads to an irreversible enhancement of channel function and blocks further enhancement of the channel by alcohols and anesthetics (Mascia et al., 2000).

In addition to the TM2-TM3 region, residues in the ECD have also been suggested to play a role in ethanol modulation. As previously mentioned, α2 receptors show reduced sensitivity to the effects of alcohol. These differences have been attributed to a single amino acid, A52. Mutation of this residue to a serine results in α1 GlyRs that show a reduced sensitivity to ethanol, similar to α2, which has a threonine in this position (Mascia et al., 1996b). PMTS experiments examining the interaction between S267 and A52 found that when ethanol bound to A52 alone, it produced an inhibitory effect, while binding only to S267 leads to potentiation (Crawford et al., 2008). This suggests that the ethanol modulation of this channel is actually a summation of positive and negative allosteric effects. These residues are thought to form a single, large binding site that can accommodate several ethanol molecules (Crawford et al., 2007). The binding of alcohols to this pocket is traditionally thought to alter the pocket size, thus stabilizing the open state conformation of the channel (Cheng et al., 2008). However, recent work involving single channel recordings shows that the binding of ethanol increases burst duration, associated with an antagonization of glycine unbinding, without affecting open probability (Welsh et al., 2009).

The exact location of this binding pocket has been the subject of some debate as the precise orientation of the contributing residues is unknown. Early mutagenesis work suggested that the binding pocket was located in a water-filled cavity between the TM domains of a single subunit (intrasubunit binding) (Mascia et al., 2000; Lobo et al., 2004; Bertaccini et al., 2005). However, modeling studies using the more recent GLIC and ELIC structures place this binding pocket at the interface between adjacent subunits (intersubunit binding) (Cheng et al., 2008; Murail et al., 2011). In the absence of a GlyR crystal structure, it is difficult to definitively identify the location of this pocket. However, the structure of an anesthetic bound to the GLIC channel was recently solved (Nury et al., 2011). Interestingly, this structure includes both an intrasubunit and intersubunit water-filled cavity connected by a small linking tunnel. The size and shape of these cavities are hypothesized to change during channel gating, suggesting that both cavities could be accessible to alcohols and anesthetics in a state-dependent manner. This is consistent with previous mutagenesis work showing that certain residues important for alcohol binding are only accessible in the open state (Lobo et al., 2004).

Intracellular mechanisms have also been suggested to modulate the effects of ethanol on the GlyR. The large intracellular loop between TM3 and TM4 is the target of phosphorylation by protein kinases A (PKA) and C (PKC) (Song and Huang, 1990; Vaello et al., 1994; Ruiz-Gomez et al., 1991). Phosphorylation by PKC was shown to modulate the channel responses to ethanol, but not to glycine (Mascia et al., 1998). G-proteins also interact with the TM3-TM4 intracellular loop (Zhu and Ye, 2005) and have been shown to modulate channel function (Yevenes et al., 2003). For example, the

degree of interaction between GlyR and the Gβγ heterodimer alters the potentiation of glycine-activated currents by alcohol (Yevenes et al., 2008). The Aguayo group has suggested that these interactions are not only important for modulating the effect of ethanol on the GlyR, but may in fact be the primary mechanism through which ethanol potentiates these channels (Guzman et al., 2009; Yevenes et al., 2010). These data highlight the complexity of the interaction between ethanol and the GlyR.

Several of the behavioral effects of ethanol are consistent with the idea that this drug enhances the inhibitory GlyR current. A common behavioral measurement of intoxication by ethanol is the loss of righting reflex (LORR), characterized by increased difficulty in standing upright. LORR can be reinstated in animals that have recovered from ethanol-induced LORR by intracerebroventricular injections of glycine and serine, the glycine precursor (Williams et al., 1995), while strychnine, a GlyR antagonist, abolished these effects. Experiments in knock-in mice carrying a point mutation at residue S267 in the α1 GlyR subunit further support the hypothesis that GlyRs are important for the motor incoordinating and anesthetic effects of ethanol (Findlay et al., 2002). These animals showed reduced LORR and motor incoordination following ethanol administration. In addition, they were less sensitive to the anticonvulsant effects of ethanol during strychnine-induced seizures. GlyRs in supraspinal regions are thought to play a role in ethanol-induced hypnosis, the loss of consciousness (Ye et al., 2009).

More recently, GlyR activity in the NAcc and VTA was demonstrated to alter the rewarding effects of ethanol. These brain regions are part of the mesolimbic dopamine pathway, which is thought to mediate the reinforcing effects of ethanol and other drugs of

abuse (Leshner and Koob, 1999; Gatto et al., 1994; Di Chiara and Imperato, 1988). Ethanol intake increases GlyR responses in the VTA in adult cells; however, in neonatal cells, ethanol decreases GlyR responses, suggesting the possibility that these channels may contribute to the development of fetal alcohol syndrome (Ye et al., 2001b). In adult animals, the direct application of glycine into the NAcc causes an increase in extracellular dopamine in this area and a concomitant decrease in voluntary ethanol intake (Molander et al., 2005), while the application of strychnine produces the opposite effect. Taurine and β-alanine also increase dopamine levels in the NAcc (Ericson et al., 2006; Ericson et al., 2010). Glycine elevation in the NAcc is thought to result in a disinhibition of the $GABA_A$ neurons that project to the VTA. This disinhibition leads to an increase in acetylcholine output from the VTA, which in turn causes an elevation of dopamine levels in the NAcc (Ericson et al., 2003; Soderpalm et al., 2009). During ethanol operant self-administration, elevated levels of taurine in the NAcc were found. The magnitude of this increase correlated with the amount of ethanol consumed (Li et al., 2008). An increase in the NAcc glycine concentration was also reported. Interestingly, this increase began several minutes prior to the presentation of ethanol, suggesting that glycine may be involved in the anticipation of reward. Systemic administration of Org25935, a selective inhibitor of the glycine transporter 1 (GlyT1), successfully increased the level of glycine and dopamine in the NAcc and decreased ethanol consumption (Molander et al., 2007). This decline in ethanol intake persisted even after the reintroduction of ethanol following deprivation, suggesting that the modulation of GlyR signaling in this region could be a therapeutic target for reducing ethanol relapse

(Vengeliene et al., 2010). Cumulatively, these data support the hypothesis that the GlyR plays an important role in several aspects of alcohol addition, including initiation, maintenance, and relapse (Vengeliene et al., 2008).

Several studies have attempted to delineate the exact role of GlyR in ethanol-mediated behaviors. Knock-out and knock-in animal studies have offered several insights and helped verify some of the *in vitro* data. However, as with all studies of this type, perturbing the system can lead to significant side effects. For example, the S267Q mutant mouse displayed a strong startle phenotype, making it difficult to compare to wild-type behaviors (Findlay et al., 2003). For the majority of GlyR studies, heterozygous animals must be used because the homozygous knock-out or mutation is fatal (Crabbe et al., 2006). However, heterozygous expression introduces the possibility of "mixed channels" and compensation within the system, both of which can obscure the contribution of the studied channel. A more direct method of investigation involves the use of highly specific GlyR modulators to investigate the behavioral effects of altering channel function.

1.4 - GlyR Pharmacology

In addition to ethanol, GlyR is modulated by a variety of other compounds. As this list is quite extensive, this section will deal with only those most relevant to the work discussed in this thesis. A full review of all known GlyR modulators has been compiled by (Lynch, 2004). The locations of some of the GlyR α1 residues that are important for channel function and modulation are illustrated in **Figure 1.2**.

1.4.1 - Zinc

Zinc (Zn^{2+}) is essential for normal neural development and function, but can be toxic at high levels. Zn^{2+} is present in high concentrations throughout the brain, although most is protein-bound (Mathie et al., 2006). However, the concentrations of free Zn^{2+} can range from 5 – 25 nM (Frederickson et al., 2006), and the concentrations in the synapse may exceed 100 μM (Vogt et al., 2000). Zn^{2+} is packaged into vesicles and released along with neurotransmitters, including glycine (Birinyi et al., 2001), suggesting that it may function as a co-factor.

The effect of Zn^{2+} on the GlyR is biphasic, making this ion a unique GlyR modulator. At low concentrations (< 10 μM) Zn^{2+} potentiates glycine responses, while at higher concentrations, it has an inhibitory effect (Bloomenthal et al., 1994). Interestingly, Zn^{2+} does not have biphasic actions on the closely related $GABA_A$ receptors.

Consistent with this biphasic behavior, two binding sites for Zn^{2+} have been identified in the ECD of the α subunit (pink residues in **Figure 1.2**). A series of histidines, a residue commonly involved in Zn^{2+} coordination on proteins, constitute the low affinity inhibitory site (Auld, 2001). The substitution of H107 and H109 with alanine residues is sufficient to abolish Zn^{2+} inhibition (Harvey et al., 1999). Threonine residues 112 and 133 are also thought to contribute to this site (Miller et al., 2005a). Mutational work suggests that this site is located between two subunits, suggesting that Zn^{2+} bound to this site inhibits GlyR function by stabilizing intersubunit interactions that must be broken before the receptor can activate. Consistent with this hypothesis, the

removal of the hydrophobic interactions near this Zn^{2+} site that stabilize the closed state bias the receptor toward activation and antagonize the inhibitory effect of Zn^{2+}. Miller et al. (2008) found that the F99A mutation turned the GlyR into a Zn^{2+}-activated channel that can open spontaneously in the absence of glycine.

Traditionally, residue D80 is thought to be the principal player in the Zn^{2+} high affinity potentiating site. While mutations to this residue can abolish Zn^{2+} potentiation (Laube et al., 2000), these effects are surprisingly ligand specific. For example, channels with the D80A mutation will not show Zn^{2+} potentiation when the channel is activated by glycine; however, currents elicited by taurine are still enhanced by Zn^{2+} (Lynch et al., 1998). Mutations at three more residues (E192, D194, and H215) also significantly reduce or abolish Zn^{2+} potentiation (Miller et al., 2005b). The potentiation of glycine-mediated currents by Zn^{2+} is thought to result from a decrease in the rates of glycine unbinding (Laube et al., 2000), while a separate mechanism is proposed for taurine-mediated currents. Recently, intracellular Zn^{2+} was also shown to potentiate channel activity (Trombley et al., 2011).

The abundance of Zn^{2+} in the CNS and the co-release of Zn^{2+} with glycine suggest that the interactions of Zn^{2+} with this channel are extremely important for channel function. Indeed, homozygous knock-in mice carrying the D80A mutation exhibit phenotypes similar to those seen in human startle disease (Hirzel et al., 2006). In addition, Zn^{2+} has also been shown to alter the effect of other GlyR modulators, most notably ethanol (McCracken et al., 2010).

1.4.2 - Volatile Anesthetics

Volatile anesthetics (VA) and several inhalants have also been shown to enhance GlyR responses (Mascia et al., 1996a; Beckstead et al., 2000). The concentration of isoflurane, a common VA, required to achieve anesthesia is three times higher when the drug is applied to the brain alone as opposed to the spinal cord (Antognini and Schwartz, 1993), suggesting that the spinal cord, in which GlyR is the dominant inhibitory ligand-gated ion channel (LGIC), is a major anatomical target of anesthetics. Several of the mutations that alter the GlyR sensitivity to ethanol also affect VAs (Mihic et al., 1997; Mascia et al., 2000). Thus, ethanol and VAs are thought to have a similar and perhaps overlapping binding site (green residues in **Figure 1.2**). Functionally, the GlyR is implicated in the immobilizing effect of anesthetics. The application of the GlyR antagonist strychnine, either intravenously or intrathecally, increases the amount of anesthetic required to induce immobility (Zhang et al., 2003).

1.4.3 - Picrotoxin

The plant alkaloid picrotoxin (PTX) has inhibitory effects on both GlyRs and GABARs, with a higher potency at the latter. It is comprised of an equimolar mixture of picrotoxinin and picrotin, both of which are equally efficacious as GlyR inhibitors (Lynch et al., 1995). On $GABA_A$ channels, PTX acts as a noncompetitive antagonist by blocking the pore (Newland and Cull-Candy, 1992). A pore-blocking mechanism has also been suggested for GlyR; however, pharmacological studies found that PTX inhibition was not use-dependent and that potency decreased as agonist concentration

increased, suggesting an allosteric mode of action (Lynch et al., 1995). Recent mutational studies support the hypothesis that PTX interacts with residues in or near the pore (Hawthorne and Lynch, 2005; Wang et al., 2007; Yang et al., 2007).

Homomeric and heteromeric GlyR channels show different sensitivities to PTX. Heteromeric receptors require greater concentrations for the inhibitory effects to be seen, and this compound is often used to determine the type of channel present in electrophysiological studies (Pribilla et al., 1992). Consistent with the hypothesized pore blocking mechanism, this difference in sensitivity between the α and β subunits is attributed to residues in the TM2 pore-lining region.

Tutin, a picrotoxane molecule that shares many structural features with picrotoxin (Kudo et al., 1984), also modulates the GlyR. Interestingly, this molecule has biphasic effects, similar to Zn^{2+}, and is not affected by mutations in the pore region, suggesting a different mechanism of action (Fuentealba et al., 2011).

1.4.4 - Strychnine

Strychnine, another plant alkaloid, is a competitive antagonist at the glycine binding site that is often used to verify the contribution of GlyRs to the overall glycine-mediated signal, as it does not affect glycine binding to its site on the *N*-methyl-D-aspartate receptor (Young and Snyder, 1973). Although this compound is often considered to be GlyR specific, at high concentrations is can also antagonize certain subtypes of $GABA_A$ (Houamed et al., 1984) and nACh channels (Matsubayashi et al., 1998; Rothlin et al., 1999). Based on several structural and pharmacological studies,

strychnine is thought to bind to a site that overlaps, but is not fully identical to, the glycine binding site (Marvizon et al., 1986; Ruiz-Gomez et al., 1990) (blue residues in **Figure 1.2**). Residues from both subunit interfaces have been implicated in strychnine binding: R131 and E157 from the (-) face and Y202 and F207 from the (+) face (Grudzinska et al., 2005). Recent modeling work based on the crystal structure of the *Aplysia* acytalcholine binding protein suggests that two strychnine molecules can co-occupy the glycine binding site. Mutations at residues that were shown to mediate single strychnine binding in this model (S158, F63, and R56) dramatically decreased the strychnine effect, while mutations at residues associated with the double-bound formation (F44, Q67, and F207) increased the response to strychnine (Brams et al., 2011).

In an attempt to identify novel GlyR modulators, substances that shared structural similarities with strychnine were tested for their abilities to modulate the GlyR (Duan et al., 2009). While none of the compounds were as potent as strychnine, this study found that caffeine, a commonly used stimulant, also weakly inhibits the GlyR.

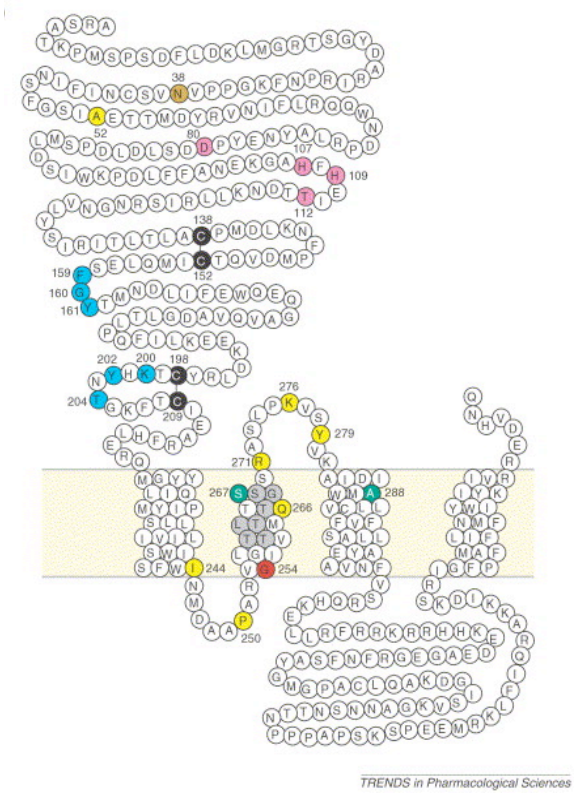

Figure 1.2: Location of functionally important GlyR α1 amino acids.

Illustration of a GlyR α1 subunit. Black residues in the ECD represent the two sets of cysteine residues that form disulfide bonds. The brown residue marks the sole N-glycosolation site for this residue. Yellow residues indicated natural GlyR mutants that result in spasmodic or hyperekplexia phenotypes. Blue residues are important for strychnine binding and agonist affinity. The green residues in TM2 and TM3 have been shown to mediate the effects of alcohols and volatile anesthetics. Pink residues mediate the modulatory effects of Zn^{2+}. The red residue in TM2 determines the main-state conductance of the channel, while the grey residues are thought to line the ion channel. *Adapted from Laube et al., 2002. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? Trends Pharmacol. Sci.* ***23:****11*

With the exception of strychnine, none of these modulators have any significant specificity for the GlyR. Thus, the ability to selectively manipulate these receptors within complex systems is severely limited. In addition, none of the currently known modulators are able to completely distinguish among the various a sub-types or a homomers and ab heteromers. These channel subtypes have distinct localizations and kinetics, suggesting that subunit-specific modulators could have differential effects. This limited pharmacology has hindered the characterization of GlyR function, leaving an important gap in our understanding of how these channels contribute to various disease states and phenotypes.

1.5 - Phage Display

In order to address the lack of GlyR-specific compounds, various approaches have been used in an attempt to identify novel GlyR modulators. These include generating modified versions of known GlyR modulators (Duan et al., 2009) and blind chemical screens (Gilbert et al., 2009). However, screens based on an existing modulator are typically limited in their diversity, while chemical screens yield a high percentage of false positives (background noise) that must be sorted through. A more efficient approach would be to use a selection system that allows for the direct isolation of compounds based on their affinity for the GlyR with a high initial diversity and the ability to limit or remove non-specific hits. Phage display (PD) can be used to identify small peptides that bind with high affinity to cellular targets. This technique utilizes bacteriophage, viruses

that infect bacterial cells and use the host machinery to replicate. Commercially available phage libraries contain billions of peptide sequences, making this a very high throughput approach. Furthermore, linking affinity selection to a biological system allows for a series of selection steps to reduce the contribution of non-specific binding.

1.5.1 - Phage Structure

While several types of phage have been used for PD, the most common is the M13 viron, which has been extensively characterized. Each individual phage consists of five surface coat proteins that encapsulate a circular single-stranded DNA, forming a long, slender rod shape (**Figure 1.3**). The majority of the phage body is comprised of pVIII proteins. These proteins have a positively charged helix on their C-termini that interacts with the encapsulated DNA (Marvin, 1998). Hydrophobic interactions allow these proteins to package together tightly, forming a long cylinder. One end of the viron is capped with pairs of the minor coat proteins pVII and pIX, while the other end is capped with pairs of pIII and pVI proteins. Proteins VII and IX are small hydrophobic proteins that are involved in the initiation of phage assembly within the host cell (Rakonjac et al., 2011). On the distal end of the phage, pIII is required for infectivity and necessary for the termination of viral assembly after infection (Rakonjac and Model, 1998).

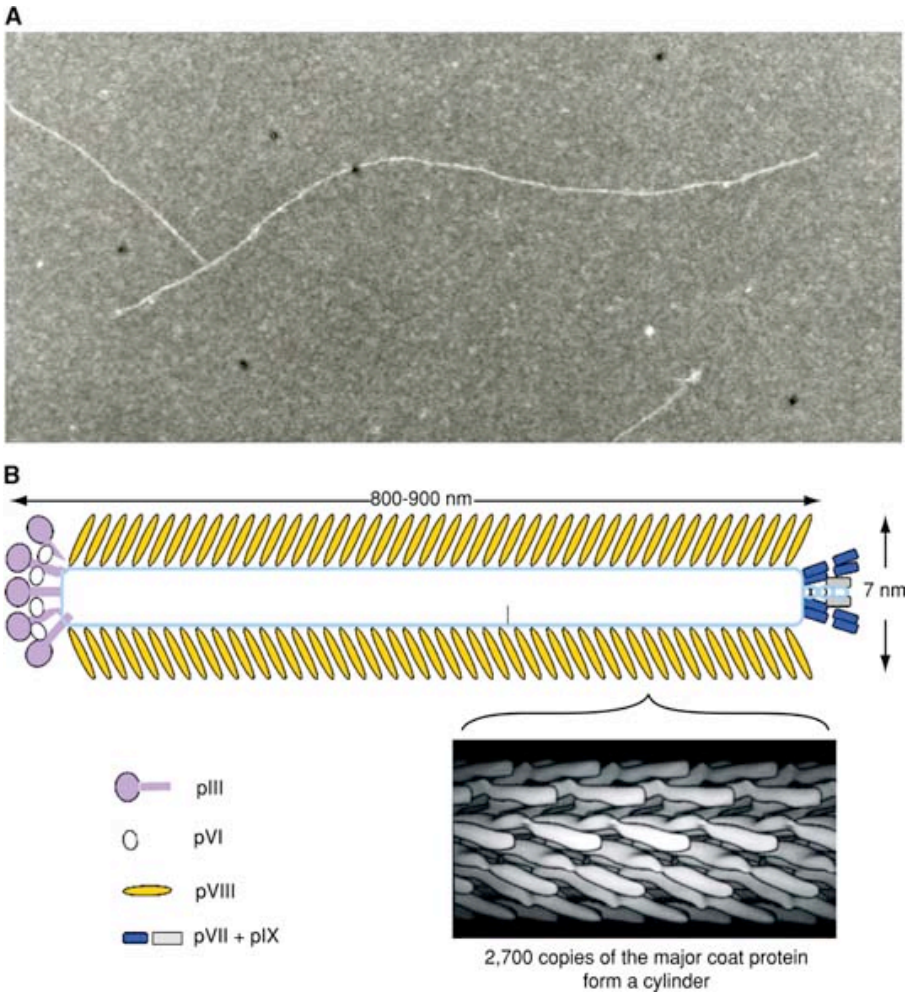

Figure 1.3: Structure of filamentous phage Ff

(A) Transmission electron micrograph of a phage (original magnification 57,000x). *(B)* Illustration of a phage showing the arrangement of the major coat protein (yellow) and the four minor coat proteins. *Inset* – Major coat protein surface domains represented as electron densities. *Adapted from Brigati et al., 2008. Phage display for generating peptide reagents. Curr. Protoc. Protein Sci.* ***15:*** *18.*

1.5.2 - Phage Replication

M13 phage infect a wide variety of gram-negative bacteria as part of their reproductive cycle. These cells are non-lytic, although infection does substantially slow down the replication of the host cell (Marvin and Hohn, 1969). Host infection involves two major steps: recognition, during which the virus binds to the bacterial cell surface receptor, and translocation, in which the viral DNA is uncoated and translocated into the bacterial cell cytoplasm. The pIII coat protein mediates the initial steps of phage replication. Protein III contains three distinct domains (CT, N1, and N2) connected by glycine-rich linkers, which allow portions of pIII to be somewhat flexible. The two amino-terminal domains (N1 and N2) are required for phage infection of a host cell and have been extensively characterized using electron microscopy and crystallography (Holliger and Riechmann, 1997; Gray et al., 1981; Holliger et al., 1999; Lubkowski et al., 1998). During recognition, the N2 domain of pIII binds to the endogenous F-pilus on the surface of the bacterial cell (Click and Webster, 1997). The pilus is then retracted into the cell membrane. This brings the phage in contact with the secondary receptor, a Tol complex comprised of TolA, TolQ, and TolR that resides in the periplasm of the bacterial membrane (Lubkowski et al., 1999). This complex is highly conserved in gram negative bacteria and is part of a larger trans-envelope Tol-Pal complex that is involved in cell division and the maintenance of cell envelope integrity (Gerding et al., 2007). The retraction cycle of the bacterial pili are spontaneous rather than a result of phage binding (Clarke et al., 2008); however, following successful infection pilus assembly is inhibited by pIII (Boeke et al., 1982), ensuring that each bacterial host is infected by only one

phage. The N1 domain of the phage interacts with the D3 domain of TolA, resulting in the entry of phage DNA into the cytoplasm and the integration of the major coat proteins into the inner membrane of the bacterial host. The exact mechanisms behind the translocation of the viral DNA into the host cell are largely unknown. However, it is hypothesized that the CT domain of pIII may be involved in the formation of a pore through which the viral DNA may pass (Glaser-Wuttke et al., 1989). Specifically, a conformation change in the CT domain may cause an uncapping of the viron, exposing the hydrophobic surfaces of pIII, pVI, and pVIII, similar to the mechanism of entry seen in eukaryotic viruses (White, 1992; Bennett and Rakonjac, 2006).

1.5.3 - Synthesis of Phage Proteins within the Host

Once inside the host cell, bacterial host enzymes synthesize the complementary (−) strand from the original ssDNA (+) strand, producing a double-stranded, covalently closed, supercoiled DNA product referred to as the replicative form (RF). RNA polymerase binds to the (+) strand, synthesizes RNA on the ssDNA template, stalls at the poly-G tract, backtracks, and finally dissociates from the template, leaving an RNA primer hybridized to the template strand (Zenkin et al., 2006). DNA polymerase III uses this primer to synthesize the (-) strand, resulting in a double stranded circle. The RF DNA then replicates to form a pool of approximately 100 RF molecules. These RF DNA molecules act as templates for the synthesis of progeny ssDNA.

The phage genome contains nine genes that encode for eleven proteins, the five structural proteins (pIII, pVI, pVII, pVIII, and pIX) and six non-structural proteins (pI,

pII, pIV, pV, pX and pXI). Transcription of the (-) strand produces the eleven coded proteins in the order in which they are required for assembly. Multiple transcripts are produced, allowing for the production of large amounts of coat proteins. The replication proteins (pII, pV, and pX) are synthesized and remain in the cytoplasm, while the structural proteins are inserted into the host membrane (Endemann and Model, 1995). The replication protein, pII, binds to a the supercoiled RF and cleaves the (+) strand, covalently binding to the 5' end (Asano et al., 1999). DNA polymerase binds to the 3' end and synthesized a new (+) strand (Meyer and Geider, 1982). In the early stages of phage infection, the new (+) strand is used to generate more (-) strands, increasing the number of RF and thus the number of translated phage proteins. This process ceases when enough pV is generated to fully coat the new (+) strands (Zaman et al., 1992). The ssDNA is fully encased in pV with the exception of a hairpin loop, which serves as the packaging signal. Protein V also inhibits the translation of pII (Michel and Zinder, 1989), thus it coordinates ssDNA production and packaging.

An assembly complex, composed of pI, pIV, pIX, and thioredoxin from the host cell, is formed near adhesion zones in the host membrane. At these locations, the inner and outer membrane of the host cell are closer together (Lopez and Webster, 1985). Protein IV spans the outer membrane, while pI and pXI form a channel that spans the inner membrane and has ATPase activity (Horabin and Webster, 1988). The amino terminal of pI and thioredoxin occupy the cytoplasmic portion (Russel, 1993). Protein VII and pIX, which will eventually cap the distal portion of the viron, plug the channel formed by pIV. This membrane-spanning complex serves as both a platform for viron

assembly and a pathway for non-lytic viral exit from the host cell. The exposed packaging signal targets the pV-ssDNA to this complex to begin viron elongation (Russel and Model, 1989).

Elongation of the phage particle requires pIII, pVI, pVII, pVIII and pIX to be embedded in the inner membrane with thioredoxin (Russel, 1991). While the exact role of thioredoxin is unknown, it is hypothesized to form a complex with pI that enhances phage production by stabilizing the binding of pI to the viral DNA (Russel, 1995). For the phage particle to assemble, pV must be stripped from the ssDNA and replaced with rings of pVIII. The hydrolysis of ATP by pI is thought to provide the energy for displacing pV from the ssDNA.

Assembly termination occurs when the entire phage ssDNA is packaged in pVIII. At this point, pIII and pIV, which are embedded in the host membrane, are added to the proximal end. Protein IV is required to stabilize the proximal end and attach pIII. A conformational shift in pIII following this attachment is suggested to be the driving force for the release of the phage from the host cell (Rakonjac et al., 1999).

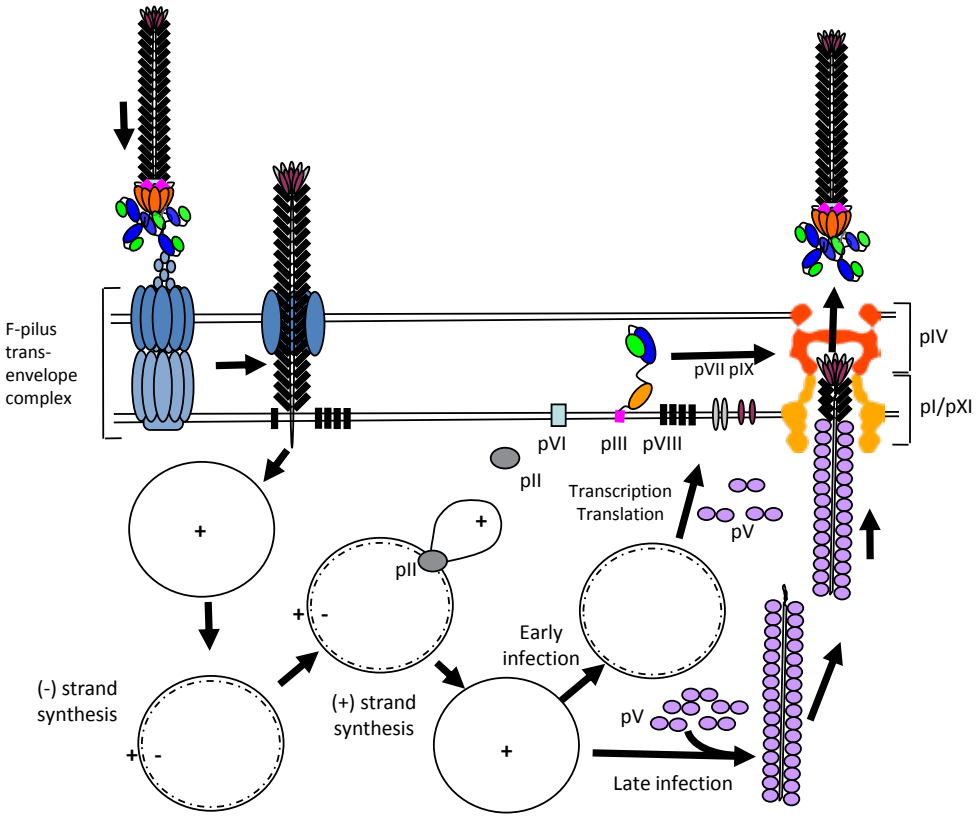

Figure 1.4: Reproductive cycle of Ff phage

Diagram illustrating the infection, replication, and release of Ff phage. Phage interact with cell surface proteins and the ssDNA of the phage is translocated into the cytoplasm of the host cell. During early infection, new positive strands are used as templates for the synthesis of negative strands, resulting in an increase in the levels of the double stranded RF DNA. These RF strands are then used to produce phage proteins. In the later stages of infection, the new positive strands are coated with pV, transported to the membrane, and packaged in pVIII and released from the host cell. *Adapted from Rakonjac et al. 2011. Filamentous bacteriophage: biology, phage display and nanotechnology applications. Curr. Issues Mol. Biol.* ***13:*** *51.*

1.5.4 – The Development of Phage Display

In 1985, it was discovered that the DNA of these organisms are able to tolerate the insertion of sequences for small peptides (Smith and Petrenko, 1997). The foreign coding sequence was spliced in-frame into a phage coat protein gene so that the coded peptide was fused to the coat protein, and thus displayed on the viron surface (Smith, 1985). This original work was done using f1 phage to express a 57 amino acid segment of EcoRI. Following replication of the virons in bacteria, the progeny were found to carry the inserted sequence. However, this display vector had several limitations. The bacterial plaques formed by the infected cells were very small, and the phage carrying the peptide insert were significantly less infective that their wild-type counter parts. These limitations arose from the placement of the peptide insert into a loop in the D2 domain of pIII, which interfered with the ability of the phage to infect bacterial hosts. A more functional library was developed using fd-tet phage in which the foreign sequence was expressed on the amino-terminal of pIII (Zacher et al., 1980). This vector has the advantage of built in tetracycline resistance. However, the DNA copy number for this system was low due to the fact that the tetracycline resistant insert disrupted the origin of the (-) strand, forcing the phage to use an alternative pathway for synthesis. Thus, infectivity in this library was low (reduced 10-fold from wild-type), which in turn reduced the phage particle yield following amplification (reduced 40-fold from wild-type).

The dominant family of phage vectors in use today is the M13mp series (Messing et al., 1977). These vectors carry an insert of *lac* DNA that encodes for the N-terminal

peptide of the enzyme β-galactosidase. This peptide segment, referred to as the α peptide, has no enzymatic activity on its own, but is capable of restoring enzymatic activity that is lost due to a specific deletion in the *lacZ* gene that removes the α domain of the N-terminus of β-galactosidase (deletion *ΔM15*). Thus, when phage carrying this vector are grown using a plating medium that contains X-gal, the chromogenic substrate of β-galactosidase, using a bacterial host carrying the *ΔM15* deletion, the plaques of infected cells are blue due to the rescue of β-galactosidase activity by the a protein, while uninfected bacterial colonies remain colorless. This allows for easy isolation of infected colonies even when the plaque size is reduced. The M13KE vector used to develop the commercially available phage libraries discussed in this thesis is derived from M13mp19.

1.5.5 – Library Diversity

The biology-based approach used in phage display has several advantages over binding assays that utilize chemical libraries. Most importantly, the genotype and phenotype remain linked throughout the amplification steps. This means that the isolation of a single phage-bound peptide will allow for the full characterization of that peptide. However, utilizing a living system also comes with some inherent disadvantages. The composition of the peptide expressed on the phage can significantly alter the phage-host interaction, and thus the ability of the phage to replicate (Pratt et al., 1966). This can lead to a bias against sequences that significantly slow the morphogenesis of the virus, as these will be selected out of the library (Rodi et al., 2002). For example, cysteine, arginine, and glycine residues are under-represented in most

commercially available libraries, while proline, threonine, and histidine are over-represented. Due to their ability to cross-link with one another, cysteine residues have the potential to form dimers with other cysteine-containing proteins in the periplasm (Haigh and Webster, 1998). In addition to the intermolecular interactions, cysteines can cross-react within a peptide, leading to misfolding. As a result, peptides carrying odd numbers of cysteine residues do not exist in these libraries (Kay et al., 1993). Proline residues, on the other hand, are often overly expressed in pIII libraries. This is possibly due to the fact that these residues instigate β-turns in the protein, a shape that is preferred over purely α-helical structures by the enzyme that removes the pIII signal sequence, signal peptidase (Malik et al., 1998). Thus, the conformational nature of the incorporated peptide can influence the inclusion of that peptide sequence into the general library. Charged residues are also a source of library bias, the most prominent being a lack of arginine residues at the beginning of the peptide. Positive charges at the amino-terminus are thought to reduce transport across the membrane (Yamane and Mizushima, 1988) and alter interactions with SecY proteins (Peters et al., 1994), which insert the pIII into the membrane (Boeke et al., 1982). Thus arginine residues are typically absent from the first position within the peptide, which lies immediately after the signal sequence.

While individual residue restrictions do impact the diversity of the library, the major factor limiting peptide expression is the redundancy of the genetic code. Certain residues are coded for by multiple codons, which biases their expression in a library built using degenerate oligonucleotides. Based on the number of redundant sequences, the diversity of any library is reduced from 100%, in which every possible sequence is

represented, to approximately 11.8%. Adding in the restrictions on individual amino acids discussed above, the diversity of a library containing 12 randomized amino acids is estimated to be around 4.4% (Rodi et al., 2002). In spite of these restrictions, each library still contains several billion sequences; however, these sequence limitations should be taken into account when analyzing phage display data.

1.5.6 - Introduction of a Randomized Peptide Insert

The foreign peptide inserted into the phage can be derived from natural sources or be a deliberately designed synthetic sequence. Most commercially available libraries are constructed using saturation mutagenesis, which allows for the highly controlled introduction of mutations into a protein sequence. This approach replaces existing codons with degenerate oligonucleotides that code for multiple amino acids. For example, the NNN codon contains all 64 natural codons. However, as previously discussed, the redundancy in the genetic code leads to heavy sequence bias in libraries constructed with this codon. In a less redundant alternative, the third codon is restricted to coding for either a G or C (NNK), resulting in 32 unique codons and a reduction in the impact of redundancy. This codon is used in the production of the libraries used in this thesis. This form of randomization is most successful when a thorough search of a localized region is required (Devlin et al., 1990; Cwirla et al., 1990). Theoretically, the diversity of the library will increase exponentially with the number of residues randomized. However, in reality the diversity is limited by factors affecting bacterial transformation, as noted above (Sidhu et al., 2000). Thus, only six or seven positions can

be simultaneously randomized in this manner with a reasonable probability of representing all possible combinations (Clackson and Wells, 1994). The libraries used for this study are predicted to have complexities on the order of 10^9. In a library with 7 randomized amino acids, this is sufficient to encode most of the possible combinations (1.28×10^9). However, in libraries carrying 12 amino acid peptides, this represents only a tiny fraction of the possible combinations (4.1×10^{15}).

To produce libraries from synthetic DNA, it is necessary to insert the DNA into a suitable vector and introduce the recombinant DNA into a bacterial host to initiate phage production. There are a variety of approaches for integrating synthetic DNA into the phage genome. The simplest involves taking advantage of the ssDNA carried by the phage. Phage ssDNA is easy to purify at a high yield and can serve as a template for oligonucleotide-directed mutagenesis (Sidhu et al., 2000). The designed oligonucleotide is annealed to the ssDNA template and enzymatically extended and ligated. This produces a dsDNA heteroplex in which one strand corresponds to the original phage DNA and the other carries the inserted peptide sequence. Original attempts to use ssDNA mutagenesis (Zoller and Smith, 1983; 1982) suffered from low mutation frequencies. This was due to the preference of the *E. coli* bacterial host for the parental (non-mutated) DNA strand. This problem was rectified following the production of template ssDNA from dut^-/vng^- *E. coli* strains (Kunkel, 1985; Kunkel et al., 1987). Template strands generated from these cells contain a significant amount of uracil bases in place of thymine bases. When dsDNA heteroplexes containing these parent strands are introduced into wild-type *E. coli* (dut^+/vng^+), the cell will preferentially inactivate the

uracil-containing parent strand, increasing the production of the synthetic strand carrying the foreign peptide sequence.

Though all of the major coat proteins have been used to develop phage libraries, expression on the pIII protein is most common. The amino terminus of N1 extends out away from the phage body in such a way that peptides expressed on this region are exposed to the environment. This accessibility enables the scanning of peptide affinity for presented targets. Although pIII plays a major role in the infection and replication process of these virons, expression at the amino-terminus places the foreign peptide away from the TolA binding site and the N2 domain (Lubkowski et al., 1999), thus infectivity is not significantly impaired.

1.5.7 – Phage Selection: "Biopanning"

Successful peptide selection relies on the ability to separate phage carrying sequences with affinity for a desired target from the general library population. This requires the target to be presented in a native form at a sufficient concentration to allow for the enrichment of binding sequences. Typically, the target of interest is immobilized, either using a solid support or adherent cell expression, and the phage library is washed on, allowing those phage carrying peptides with affinity for the target to bind. Following a series of washes to remove the non-binding phage, the bound phage can be eluted using a competitive ligand for the target or exposure to low pH, to which the phage are resistant. The collected phage can then be amplified in bacteria, allowing for several rounds of selection. The insertion of the peptide sequence into the phage genome

establishes a link between the genotype and phenotype of these phage. Each phage will pass on its entire genome, including the foreign peptide sequence, to its progeny. This enables phage to be used for several rounds of selection and amplification, an important advantage over chemical-based affinity selections. However, amplification can introduce a replication bias. That is, phage carrying peptides that randomly increase their infectivity or any insertless phage left over from the original library will grow more quickly. This is particularly problematic for phage libraries with induced structural constraints. Thus, the selection process is a mixture of display level and binding affinity.

1.5.8 – Utilization of Phage Display

PD has been used in a variety of fields to identify binding motifs, cell-specific peptides, and peptides that bind to and modulate several cellular proteins. In addition to fully randomized peptides, libraries based on existing proteins have also been developed. The first proteins to be cloned for PD were antibody fragments (McCafferty et al., 1990). Both "natural" antibodies derived from donor B cells (Clackson et al., 1991) and synthetic antibodies in which a random sequence has been inserted into the complementary determining region (Hoogenboom and Winter, 1992) have been used for successful PD selection.

Libraries based on known ligands have also been developed. This approach typically involves introducing random mutations throughout the ligand (Cain et al., 2000), introducing targeted mutations to specific regions of the ligand (Cabibbo et al., 1995), or constructing a library by shuffling through sections collected from a

homologous family (Stemmer, 1994). This technique was recently used to identify a scorpion toxin-based modulator of the Kv1.3 channel (Takacs et al., 2009). Different versions of PD have been used to identify therapeutic recombinant antibodies (Bradbury and Marks, 2004), modify the properties of known proteins (Rebar and Pabo, 1994), and identify peptides that target specific tissue or cell types (Pasqualini and Ruoslahti, 1996).

Most relevant to this work, PD based on random peptide sequences has been used to isolate peptides that show affinity for ion channel targets and modulate their function. Some of the most striking examples include agonists that show nanomolar potency at the erythopoetin (Wrighton et al., 1996), thrombopoetin (Cwirla et al., 1997), and fibroblast growth factor receptors (Ballinger et al., 1999). In addition, peptides have been identified that bind to the selected target and alter the effects of other modulators. For example, Balass et al. identified a peptide that binds to the nAChR at the α-bungarotoxin binding site, thus preventing the binding of this toxin (Balass et al., 1997). A peptide that acts as a non-competitive inhibitor of the *N*-methyl-D-aspartate receptor was also identified using PD (Li et al., 1996). The successful application of PD in the identification of channel modulators supports the hypothesis that PD can be used to identify peptides that will bind to and modulate the GlyR.

1.6 - Channel Modulation

As the bulk of this thesis will deal with the identification and characterization of compounds that modulate channel function, a brief discussion of the terms and mechanisms associated with channel modulation is warranted.

The discussions here will largely deal with two distinct classes of agents: those that bind to and directly activate the channel (orthosteric agents) and those that merely modulate the function elicited by the primary agonist (allosteric agents). In general, orthosteric agents produce significant perturbations of the transduction mechanism (leading to channel opening), while allosteric agents fine-tune the corresponding neurotransmission without opening the channel on their own (Maksay, 2009). There are, of course, grey areas in this field, and what exactly constitutes an orthosteric modulator versus an allosteric modulator is the subject of some debate (Schwartz and Holst, 2007). For example, a recent report demonstrated that α7 nACh receptors can be activated following binding to what has been traditionally recognized as an allosteric site (Gill et al., 2011). However, for the purposes of this thesis, a modulator that can produce activation of the channel on its own will be referred to as an orthosteric ligand, while a modulator that has no effect on its own but modulates the effect elicited by another ligand will be referred to as an allosteric modulator.

As previously discussed, channel activation involves the binding of a ligand that triggers a conformational wave ultimately leading to gating and channel opening. While an explanation of the detailed mechanisms of this effect are beyond the scope of this thesis, it is important to note the major contributors. All ligands, and indeed all channel modulators of any type, have two major characteristics that determine the magnitude of their effects: affinity for the target and efficacy observed after binding. Affinity refers specifically to the tightness of binding of the compound to the receptor, while efficacy refers to the ability of the compound, once bound, to have a functional effect, typically by

altering the structure of the channel in some way. Together these components cause a change in receptor function, and an increase or decrease in either will alter the observed effect. Affinity and efficacy are intimately linked, and determining if an allosteric modulator affects the affinity or the efficacy of a given orthosteric ligand is incredibly difficult (Colquhoun, 1998). In this thesis, the preliminary selection process, phage display, gives a measure of affinity only. The peptides are selected purely on their ability to bind to the receptor, regardless of the effect on channel function. However, the estimated affinity from this selection process may or may not relate to the final modulatory effect seen in the functional assay, as this response is a combination of affinity and efficacy.

1.7 – Chapter Overview

The remainder of this dissertation will be divided into four chapters. Chapter 2 details the materials used in these experiments as well as the methods applied and the data analysis procedures. The primary techniques employed are phage display panning and whole-cell two-electrode electrophysiology.

Chapter 3 describes the results of the experiments used to test the hypothesis that phage display can be used to identify peptides that bind to the GlyR. Phage display has been successfully used to isolate peptides that have affinity for ion channels and receptors. We developed a modified phage panning protocol to allow for the selection of peptides that bound to the extracellular domain of the α1 GlyR, and several peptides were identified using this approach. In addition, these experiments also tested the hypothesis that some of these phage-derived peptides would have modulatory effects on GlyR function. Using two-electrode physiology, we found several peptides that modulated the glycine responses of α1 GlyR, some with high specificity.

Chapter 4 describes the results of experiments in which the specificity of the panning procedure was increased. These experiments tested the hypothesis that the phage display protocol used to identify peptides that bind to and modulate α1 GlyR can be modified to bias selection toward peptides that differentiate between GlyR subtypes. By including two GlyR subtypes (α2β and α3β) in the negative selection portion of the panning procedure, we identified several peptides that show preferential modulation of α1β Gly channels.

Chapter 5 describes experiments designed to test the hypothesis that a common peptide contaminant, trifluoroacetic acid (TFA), also has modulatory effects on GlyR function. It was determined that TFA not only alters the response to glycine in these channels, but does so in a subtype-specific manner.

Chapter 6 is an overall discussion of the results of the experiments outlined in the preceding chapters. General conclusions regarding the utility of the phage protocol and peptides used in these studies as well as proposed directions for future studies are outlined in this section.

2.0 | MATERIALS AND METHODS

This chapter contains detailed descriptions of the experimental procedures and materials used in the following chapters. A summary of the methods used for individual experiments can be found in the methods section of the appropriate chapter.

2.1 - Preparation of cDNA Clones

The cDNA for human GlyR α1 and β subunits and $GABA_A$ α1 subunit were previously subcloned into a modified pBK-CMV vector lacking the coding for the *lac* promotor and the *lacZ* start codon. The plasmid contains a cytomegalovirus (CMV) promotor as well as a gene conferring resistance to the antibiotic kanamycin and enables the expression of these subunits in heterologous expression systems. The cDNA for the α2 subunit was cloned in the laboratory of Dr. Neil Harrison and placed into the pCSII vector, which contains a gene for ampicillin resistance. The $GABA_A$ β2 and γ2 subunits and the $GABA_C$ ρ1 subunit were also cloned into this vector by Paul Whiting at Merck. The rat α3 subunit was cloned into the pTracer-CMV2 vector in the laboratory of Dr. Heinrich Betz. The cDNAs were isolated from XL1-Blue *E. coli* (Stratagene) transformed with the respective GlyR plasmid. Bacterial colonies were then grown on agar plates containing 50 mg/ml kanamycin in order to select for successfully transformed cells. Individual colonies from these plates were grown in LB broth overnight in a shaker incubator. GlyR cDNA was then isolated from the bacterial cells using a HiSpeed Plasmid Maxi Kit following the manufacturer's protocol (Qiagen,

Valencia, CA). The concentration and quality of the collected plasmids were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples with an A_{260}/A_{280} ratio near 1.8 were considered suitable for use.

2.2 - Site Directed Mutagenesis

Custom oligonucleotide primers were designed using the NetPrimer primer design program (Premier Biosoft) containing the desired mutations and obtained from Integrated DNA Technologies (Coralville, IA). Primers were designed to be 35 – 45 base length sequences with more than 60% GC content, a melting point (T_m) greater than 78^{o}C, and a minimal possibility for intraplasmid loop formation (Liu and Lovinger, 2000). All mutants were made using the a1 GlyR cDNA as the template and the Stratagene (Cedar Creek, TX) QuickChange Mutagenesis Kit. The forward and reverse primers (125 ng/ml) were individually combined with the template a1 cDNA (25 ng/ml), nucleotide tri phophates, and Taq DNA polymerase and run through the first 15 cycles of a thermocycling protocol in a PTC-100 thermocycler. The forward and reverse mixtures were then combined 1:1 and run together for the remainder of the protocol (additional 15 cycles). Linear amplification was used: denaturing of template cDNA at 95^{o}C, annealing of the primer at 55^{o}C, extension from the 3' end at 68^{o}C. The cycle was repeated a total of 30 times and then the temperature was held at 4^{o}C. To isolate the newly mutated DNA, the entire product was then digested with the *DpnI* restriction enzyme to remove the template DNA. These new plasmids were then transformed into XL1-blue *E. coli* as

described above. Samples of the amplified DNA were sent for sequencing using a dideoxy fluorophore method to verify the incorporation of the desired mutation.

2.3 - HEK Cell Culture

Human Embryonic Kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were stored in liquid nitrogen. Before they were used in the phage panning, cells were thawed and grown according to standard procedures (Freshney, 2002) for three cycles. Cells were cultured with Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate supplemented with 10% fetal bovine serum, Glutamax, and PenStrep (Invitrogen, Carlsbad, CA) and kept at 37^{o}C in a 5% CO_2 atmosphere. Cell lines were split every 5 days with 5% trypsin/EDTA in Hank's balanced salt solution (Invitrogen) for 25 cycles, after which new aliquots of early passage cells were started. Cells were transfected with 8 mg of GlyR cDNA using either the PolyFect reagent (Quiagen, Valencia, CA) or the Lipofectamine2000 reagent (Invitrogen). Untransfected control cells were exposed to the transfection reagent in the absence of GlyR cDNA. All cells were incubated for 48 h prior to use.

2.4 - Phage Display Panning

Phage display libraries expressing peptides that were 12 (D12) or 7 (D7) amino acids in length were obtained from New England Biolabs (Ipswitch, MA) and stored at -20^{o}C.

On panning day one, the first negative selection plate (untransfected HEK cells for the D12 library or α2β/α3β GlyR expressing plates for the D7 library) was washed three times with 0.01 M phosphate-buffered saline (PBS) containing 8.2 mM $NaPO_4$, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl with 1.5% bovine serum albumin (BSA) and 0.1% Tween-20 (PBS/BSA-T). A 10-mL aliquot of the phage library (containing 2 x 10^{11} phage particles) was diluted in 1 ml of PBS/BSA-T and applied to the negative control plate. The cells were incubated with the phage at room temperature for 30 min with continuous rocking. Phage that did not bind in the negative control plate were removed with a pipette. For the experiments using the D7 library, these phage were applied to the second negative control plate expressing the control channel that was not expressed in the first negative control plate (α2β/α3β) and the negative selection process was repeated. The non-binding phage were then applied to a plate of target-expressing cells (GlyR α1 for the D12 experiments, GlyR α1β for the D7 experiments) and incubated for 60 min at room temperature with constant rocking. The non-binding phage from this positive selection were discarded, and the plate was washed five times with PBS/BSA-T. The bound phage were eluted by lowering the pH using 0.2 M glycine HCl (10 M HCl buffered to pH 2.2 with glycine) with 1 mg/ml BSA. Plates were incubated with this elution buffer for 10 min at room temperature. The eluate was removed and neutralized with 150 ml of 1M Tris-HCl (pH 9.0). Samples of the eluate were taken for titering (10 ml), and the remainder was added to 20 ml of *E. coli* (A_{600}) in LB broth for amplification. After 4.5 h of incubation, the bacterial culture was transferred to a 50-ml Falcon centrifuge tube and spun at 10,000 rpm for 10 min at 4°C. The supernatant was

transferred to a fresh tube and respun. The upper 80% of the supernatant was again transferred to a fresh tube, and a one-sixth volume of polyethylene glycol (PEG)/NaCl (20% w/v PEG 8000 and 2.5 M NaCl) was added. Phage were allowed to precipitate overnight at 4°C.

On panning day two, the PEG precipitates were spun at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the precipitate was spun again. Residual supernatant was removed using a pipette. The pellet was resuspended in 1 ml of Tris-buffered saline (50 mM Tris-HCl pH 7.5 and 150 mM NaCl), transferred to a 1.7-ml microcentrifuge tube, and spun at 10,000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube, and the suspended phage were precipitated with PEG/NaCl on ice for 60 min. The phage were spun at 10,000 rpm for 10 min at 4°C, and the supernatant was discarded. The pellet was resuspended in Tris-buffered saline and spun again for 1 min. The supernatant was then transferred to a fresh tube and stored at 4°C. Aliquots of the amplified phage were placed in bacteria (A_{600}) and titered at three consecutive dilutions.

For successive panning rounds, the amplified phage were diluted to 2×10^{11} in 1 ml of PBS/BSA-T. If the amplified phage count was lower than 10^{11}, dilutions as low as 10^9 could be used. After several panning rounds (five for the D12 library, seven for the D7 library), individual plaques from the most recent titer plates were isolated and incubated overnight in 1 ml LB broth at 37°C with agitation. The overnight culture was then purified using the S.N.A.P. MiniPrep kit (Invitrogen) according to the manufacturer's instructions. The phage DNA was sequenced in-house using a 96gIII

sequence primer (New England Biolabs), and the sequences of the fusion peptides were recorded. Peptides were synthesized by Peptide 2.0 Inc (Chantilly, VA).

2.5 - Oocyte Isolation and Injection

Xenopus laevis frogs purchased from Xenopus Express (Homosassa, FL) were housed at 19^{o}C on a 12-hour light/dark cycle. The oocytes were surgically removed from anesthetized animals via a small incision in the lower abdomen. Following surgery, the incision was sutured closed and the frog was allowed to recover. Each animal was used for up to three surgeries.

Prior to injection, three outer membranes must be removed from the oocytes. Stage V and VI oocytes were placed in a hypertonic isolation solution, which allowed for the separation of the outer oocyte membranes so that the thecal and epithelial layers could be removed using forceps. The oocytes were then exposed to a 0.5 mg/ml type 1A collagenase buffer (Sigma) for 15 min to remove the follicular membrane. The isolated oocytes were then placed in an isotonic solution, modified Barth's saline (MBS).

The cDNAs used for oocyte injections were prepared as described above. cDNA stocks were diluted to 50 ng/ml in sterile deionized water (diH_2O). A capillary glass injector tip was made using a Sutter Instruments P-30 Flaming/Brown puller. The injector was backfilled with mineral oil and placed on a Drummond Nanoject II injector. This injector was used to front load 1 ml of the diluted cDNA. Using the "blind" injection method of Coleman (1984), cDNA was injected into the animal poles of the oocytes. Injected oocytes were incubated overnight at 19^{o}C in 96-well plates containing

incubation media that had been sterilized using a 0.22-mm filter. Expression was typically seen within 48 h, and all electrophysiological data were recorded within 2-6 days of cDNA injection.

2.6 - Two-electrode Voltage-clamp Recordings from *Xenopus* Oocytes

Currents were recorded from *Xenopus* oocytes heterologously expressing GlyRs or GABARs using a Warner Instruments OC-725C Oocyte two-electrode voltage clamp. Two high resistance (0.5 – 10 MW) electrodes were pulled from capillary glass (FHC) using a Sutter Instrument P-30 puller and then filled with 3 M KCl. Solutions of MBS or MBS + drug (pH 7.5) were perfused into the chamber at a rate of 2 ml/min using a peristaltic pump (Cole Parmer Instrument Co, Vernon Hills, IL). Excess fluids were removed from the chamber by a waste line. Oocytes were placed in a small well within the recording chamber and impaled in the animal pole with both electrodes. One electrode measures the voltage of the oocyte membrane, while the other injects the necessary current to maintain the desired potential (-70 mV). When the channels expressed by the oocyte open, the channel allows chloride ions to flow outward. This flux is detected by the voltage electrode as a depolarizing force, which causes the current electrode to inject negative current in order to hold the command voltage. This feedback occurs continuously and the output (the injected current required to counteract the flux of chloride ions) is recorded using either a paper chart recorder (Cole Parmer) or digitized using a PowerLab 4/30 digitizer (ADInstruments) and recorded on a PC laptop (Dell) using the LabChart recording program.

2.7 - Analysis of Macroscopic Currents

The peak amplitudes of the macroscopic currents resulting from GlyR or GABAR activation were either measured manually from chart recorder paper or digitally from the LabChart file. These values were corrected for the baseline current and recorded in an Excel spreadsheet (Microsoft, Redmond, WA). Peptide or drug effects were calculated as percent changes compared with the effects produced by glycine or GABA alone. These values were grouped by condition and the mean and standard error of the mean were calculated. To determine statistical significance, the percent enhancement or inhibition values obtained in the presence of peptide or drug were compared by either a t-test or one- or two-way analysis of variance, as indicated, with a criterion of $p < 0.05$ signifying a statistically significant effect.

3.0 | IDENTIFICATION OF A NOVEL ALLOSTERIC MODULATOR OF THE GLYCINE RECEPTOR USING PHAGE DISPLAY

3.1 - Introduction

The glycine receptor (GlyR) is a member of the Cys-loop superfamily of ligand-gated ion channels, which also includes the γ-aminobutyric acid type A ($GABA_A$) and serotonin-3 receptors (Ortells and Lunt, 1995). GlyRs constitute the major inhibitory neurotransmitter receptor system in the brainstem and spinal cord, where they are thought to play a role in the modulation of pain signals and the effects of volatile anesthetics. GlyRs are also found throughout higher brain regions, including the thalamus, hippocampus, and nucleus accumbens, where they were recently shown to be involved in the reinforcing properties of ethanol (Lynch, 2004; Molander and Soderpalm, 2005). The GlyR is only one of many ion channels and receptors thought to play a role in pain perception, the effects of alcohol and volatile anesthetics, and in determining the state of neuronal excitability. The isolation of the contribution of the GlyR to these various functions is hindered by the fact that, to date, no potent and efficacious allosteric modulators that act specifically at the GlyR have been identified.

One method for identifying molecules that show affinity for a selected target is phage display (PD). PD involves the expression of a random library of small peptides on the coat proteins of bacteriophage. This approach has been used to successfully identify peptides that bind with high affinity to a wide variety of cellular targets. In this thesis, I combined PD technology with standard electrophysiological testing to identify peptides

that modulate GlyR function without affecting two closely related GABA receptors**.** I utilized the Ph.D.™-12 Phage Display Peptide Library (New England Biolabs), which is based on a combinatorial library of random dodecapeptides fused to a minor coat protein (pIII) of M13 phage. The library consists of approximately 2.7 x 10^9 electroporated sequences amplified once to yield approximately 100 copies of each sequence in 10 µl of the supplied phage, making this a very high-throughput approach for identifying GlyR-binding peptides. The goal of these experiments is to develop a phage selection protocol that will allow for the identification of peptides that bind to the GlyR and modulate its function.

3.2 - Materials and Methods

Phage selection as well as the isolation, injection, and two-electrode voltage clamp of *Xenopus* oocytes were conducted as described in Chapter 2. Specific methods pertaining to the experiments in this chapter are outlined below. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Human Embryonic Kidney cells (HEK 293) were grown according to standard procedures (Freshney, 2002). Cells were cultured at 37^{o}C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate, and 10% fetal bovine serum (Invitrogen). Cell lines were split every 5 days with trypsin/EDTA in Hank's balanced salt solution. Cells were transfected with 8 mg of GlyR α1 subunit cDNA using PolyFect reagent (Qiagen). Control cells (untransfected HEK cells) were

exposed to PolyFect and then split with no DNA exposure. All cells were incubated for at least 48 h before use in panning.

Panning day 1: A plate of control cells was washed three times with 0.01 M phosphate-buffered saline (PBS) containing 8.2 mM $NaPO_4$, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl with 1.5% bovine serum albumin (BSA) and 0.1% Tween (PBS/BSA-T). An aliquot containing 2 x 10^{11} phage from the D12 phage library (New England Biolabs) was diluted in 1 ml of PBS/BSA-T. Phage were then applied to control cells and rocked gently at room temperature for 30 min. Phage that did not bind in this negative selection procedure were removed from the plate with a pipette, applied to a plate of GlyR-expressing cells (positive selection), and rocked gently at room temperature for 60 min. Non-binding phage were discarded, and the plate was washed five times with PBS/BSA-T. Elution of the bound phage was performed by lowering the pH using 0.2 M glycine HCl (10 M HCl buffered to pH 2.2 with glycine) plus 1 mg/ml BSA and rocking the cells at room temperature for 10 min. The eluate was removed and neutralized with 150 ml of 1 M Tris-HCl (pH 9.0). Titering was performed, and the remainder of the eluate was added to 20 ml of *Escherichia coli* (A_{600}) in LB broth. After 4.5 h or incubation, the culture was transferred to a 50-ml Falcon centrifuge tube and spun at 10,000 rpm for 10 min at 4^{o}C. The supernatant was transferred to a fresh tube and respun. The upper 80% of the supernatant was again transferred to a fresh tube and a one-sixth volume of polyethylene glycol/NaCl (20% w/v polyethylene glycol 8000 and 2.5 M NaCl) was added. Phage were allowed to precipitate overnight.

Panning day two: The polyethylene glycol precipitates were spun at 10,000 rpm for 15 min at 4°C. The supernatant was decanted, and the precipitate was spun again. Residual supernatant was removed using a pipette. The pellet was resuspended in 1 ml of Tris-buffered saline (50 mM Tris-HCl pH 7.5 and 150 mM NaCl), transferred to a 1.7-ml microcentrifuge tube, and spun at 10,000 rpm for 5 min at 4°C. In a fresh tube, the suspended phage were reprecipitated with polyethylene glycol/NaCl on ice for 60 min. After spinning at 10,000 rpm for 10 min at 4°C, the supernatant was discarded. The pellet was resuspended in Tris-buffered saline and spun again for 1 min. The supernatant was then transferred to a fresh tube and stored at 4°C. Aliquots of the amplified phage were titered at three successive dilutions.

For successive panning rounds, the amplified phage were diluted in PBS/BSA-T so that the input concentration was always 2×10^{11} virons. At the end of five rounds of panning, individual plaques from the most recent titer plates were isolated and incubated overnight in LB broth at 37°C with agitation. The overnight culture was then purified using the S.N.A.P. MiniPrep kit (Invitrogen) and the phage DNA was sequenced in-house using a -96gIII sequencing primer (New England Biolabs). Individual peptide sequences were sent to Peptide 2.0 Inc. (Chantilly, VA) for synthesis. Peptides were received as lyophilized powders of 98% pure TFA salts and were dissolved in MBS at a concentration of 100 mM. These stock peptide solutions were stored at 4°C.

Two high-resistance (0.5 – 10 MW) glass electrodes filled with 3 M KCl were used to impale the animal poles of isolated oocytes injected with either GlyR α1 or GABA cDNA. Cells were voltage-clamped at -70 mV using a Warner Instruments OC-

725C oocyte clamp (Hamden, CT), and MBS was perfused over them at a rate of 2 ml/min using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co, Vernon Hills, IL) through 18-gauge polyethylene tubing. All glycine, GABA, and peptide solutions were prepared in MBS, and all applications (30 sec) were followed by 5 – 10 min washout periods as appropriate.

Current peak responses were measured from chart recorder tracings. Peptide effects were calculated as percent changes compared with the effects produced by glycine or GABA in the absence of peptide. In all cases, oocyte data were obtained from at least two different frogs. The percent enhancement or inhibition values obtained in the presence of peptide were compared by either one- or two-way analysis of variance, as indicated, to determine statistical significance, with a criterion of $p < 0.05$.

3.3 - Results

3.3.1: Development of the Phage Display Selection Assay

PD selection requires that the target be purified, immobilized, and presented in the correct conformation. With soluble proteins, the target is often attached to a scaffold to immobilize it for the selection process. While this approach does allow for selection against the entire protein surface, it is not ideal for membrane-bound proteins, such as ion channels. The presence of the cell membrane is crucial to the folding and subsequent functionality of channel proteins (Heidmann et al., 1980). In addition, the presence or absence of other cellular components can alter the insertion of the channel into the

membrane. Another approach is to use the isolated extracellular domain of the target (Kallen et al., 2000; Saggio et al., 1995); however, this is limited to targets that can be easily purified and correctly folded in the absence of transmembrane domains. The ideal selection process for ion channel targets would involve the expression of the channel in a system that ensures the proteins are in the same conformation as those found *in vivo*. However, the use of cell-expressed channels, such as those found in primary neuronal culture, introduces a complex background of other cellular components that can hinder the ability to isolate phage bound to the target of interest. Electrophysiological studies aimed at characterizing ion channels have often utilized the expression of the channel of interest in non-neuronal cell lines, such as human embryonic kidney cells (HEK) or Chinese hamster ovarian cells (CHO). These cells possess the necessary machinery to generate functional channels and insert them into the cell membrane. We chose HEK cells for the expression of GlyRs in this study. GlyRs expressed in HEK cells have been used in a wide variety of experimental assays. However, HEK cells contain a variety of endogenous membrane components. In order to remove the phage from our library that bind to these components, we used a modified negative selection protocol (Oyama et al., 2006). The phage were first applied to a plate of HEK cells that had been treated with the transfecting agent in the absence of GlyR DNA (blank cells). The phage that bound to these cells, i.e., those carrying peptide sequences with affinity for the endogenous HEK membrane components, were discarded. The phage that did not bind were siphoned off and applied to a second plate of cells. These cells were exposed to the same transfecting reagent in the presence of GlyR α1 DNA. Thus, they contained homomeric α1 GlyRs in

addition to all of the membrane components found in the first plate of cells. The phage that bound to these cells were collected for further rounds of amplification and panning, as outlined in Materials and Methods. It is important to note that this selection process limited the sequences collected to only those peptides that bound to the extracellular portion of the channel. Homomeric GlyR α1 was chosen as the target rather than heteromeric GlyR α1β because of its invariant stoichiometry: heteromeric α1β receptors contain αα, αβ, and βα intersubunit interfaces. In addition, we wanted to avoid the possible complication that some receptors in our population were α1 homomers, while others were α1β heteromers. After five rounds of panning, 35 individual colonies were sequenced to identify the peptides inserted in the phage pIII protein **(Table 3.1)**. Three sequences were harvested from multiple colonies, implying selective enrichment of our phage pool. No clear amino acid sequence homology was seen among the collected sequences. Due to the size of the extracellular domain of the GlyR, and thus a large number of potential binding sites, this diversity of peptide sequences was not unexpected.

Phage	Sequence												Frequency
12-100	T	S	Y	T	T	S	I	F	G	P	R	A	1
12-101	I	L	A	N	D	L	T	A	P	G	P	R	1
12-104	N	Y	M	S	A	L	A	I	T	T	S	L	1
12-105	K	P	A	N	L	T	S	T	P	W	V	P	1
12-106	L	L	A	D	T	T	H	H	R	P	W	T	1
12-107	I	S	M	P	V	R	P	L	L	Q	D	F	1
12-108	S	I	V	S	T	Q	T	S	L	P	L	N	1
12-109	S	M	T	S	H	N	Q	W	H	L	L	A	1
12-110	A	N	P	S	P	S	T	H	H	L	T	P	1
12-111	T	T	T	L	L	T	A	T	P	H	P	H	1
12-112	Y	P	S	F	T	H	S	A	T	P	S	L	1
12-113	F	H	Q	N	S	L	R	V	H	S	S	P	1
12-114	Q	D	V	H	L	T	Q	Q	S	R	Y	T	1
12-115	N	L	N	H	E	R	S	Q	N	L	K	M	1
12-116	Y	E	S	I	R	I	G	V	A	P	S	Q	1
12-117	E	R	V	M	L	P	F	P	P	A	P	W	2
12-119	I	P	W	T	Q	H	M	A	M	S	P	M	1
12-120	T	N	T	S	W	M	T	A	M	T	P	F	1
12-121	T	H	T	T	N	A	E	G	Y	S	P	V	1
12-122	T	M	G	F	T	A	P	R	F	P	H	Y	1
12-124	S	V	S	V	G	M	K	P	S	P	R	P	4
12-123	S	I	N	G	Q	W	M	R	A	I	G	K	1
12-125	G	I	Q	L	A	N	P	P	R	L	Y	G	1
12-127	Q	D	M	L	K	P	Y	V	D	P	L	H	1
12-128	S	H	H	I	P	S	Y	Q	W	P	L	H	1
12-130	W	A	E	T	W	P	L	A	Q	R	P	P	1
12-131	S	N	Q	T	S	D	R	P	P	L	L	T	1
12-133	S	S	L	E	P	W	H	R	T	T	S	R	2
12-136	E	W	L	A	Y	D	G	I	R	A	Y	S	1
12-138	E	T	L	P	I	T	L	I	A	R	L	T	1

Table 3.1: Sequences of peptides identified by panning against α1 GlyR

A total of 35 sequences were collected following five rounds of panning. Frequency refers to the number of times a particular sequence was collected. The diversity of the peptide sequences was high, with no clear regions of homology.

3.3.2: Functional Characterization of Selected Peptides

Ten peptide sequences were chosen from this list to be tested for their ability to modulate GlyR function. Two-electrode voltage clamp in *Xenopus* oocytes was used to characterize the functional effects of these peptides on homomeric GlyRs. A maximally effective concentration of glycine (10 mM) was first applied to determine the level of GlyR expression. This maximal response was used to determine a low concentration of glycine that elicited a response equal to ~10% of the maximal current (EC_{10}, where EC is the effective concentration). The EC_{10} was applied several times to ensure a stable baseline glycine response. The oocyte was then incubated with 30 mM of a peptide for 30 s, followed by co-application of the peptide and EC_{10} glycine. Peptides that did not show a significant effect at this concentration were not tested further. This does not rule out the possibility that effects might be seen at higher concentrations; however, peptides requiring more than 30 mM would not be as useful as those that were more potent. None of the peptides tested had any effect in the absence of glycine. This implies that they neither function as agonists at the glycine-binding site nor affect the function of any other naturally-occurring oocyte proteins that might influence the holding current. Some peptides, such as D12-106, inhibited the effect of glycine (**Figure 3.1A**), while others, such as D12-105, enhanced the glycine effect (**Figure 3.1B**). The ten peptides tested exhibited a variety of effects on GlyR function, ranging from 65% inhibition to 124% potentiation of the effects of EC_{10} glycine (**Figure 3.1C**).

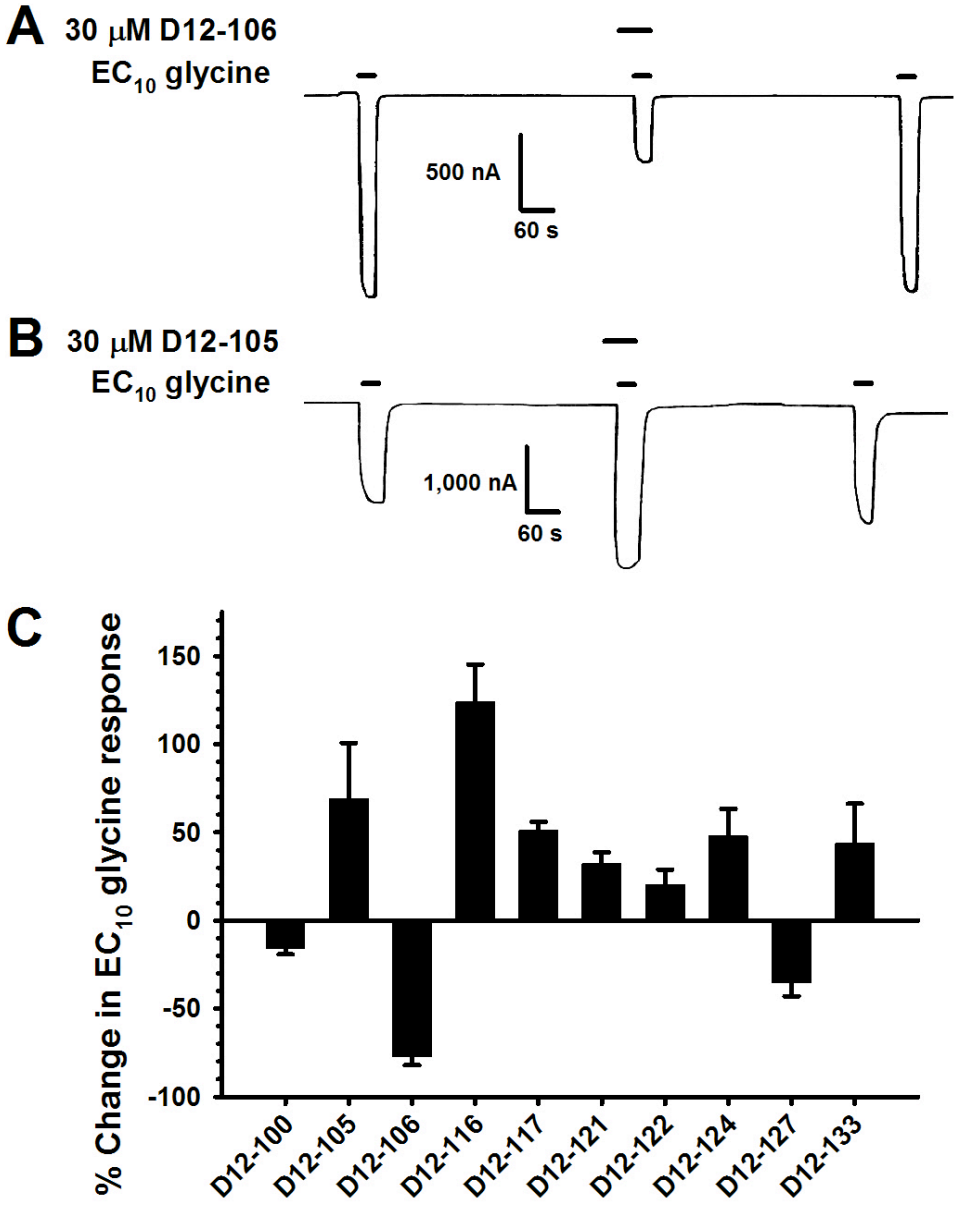

Figure 3.1 – Identification of peptides acting as allosteric modulators at the GlyR

(A, B) Representative tracings showing the effects of a 30 μM concentration of peptide D12-106 *(A)* and D12-105 *(B)* on the currents elicited by EC_{10} glycine. Neither peptide had any effect in the absence of glycine. When co-applied with glycine, D12-106 inhibited the effect of EC10 glycine *(A)*, while D12-105 potentiated GlyR function *(B)*. *(C)* Percent changes in EC_{10} glycine responses produced by co-application with 30 μM of D12 peptides following a 30 s application of peptide alone. These peptides exhibited varying degrees of potentiation or inhibition at the GlyR, but none acted as agonists by directly activating the receptor. Data are expressed as the mean ± S.E.

3.3.3: Test for Specificity using $GABA_{A/C}$ Receptors

Although the peptides were chosen based on their abilities to bind to the GlyR, there was a chance that they might also affect other structurally related channels. The members of the Cys-loop receptor superfamily possess considerable structural and amino acid sequence homology (Ortells and Lunt, 1995). The GlyR is most similar to the other anion-conducting channels in this family. Of these, the $GABA_C$, previously referred to as GABA ρ1, subunit is the most similar to the GlyR α1, with ~60% amino acid sequence identity, followed by the other members of the $GABA_A$ group (Grenningloh et al., 1987a). To test the specificity of our peptides, we compared the functional effects on the GlyR α1 receptors with those on homomeric GABA ρ1 and heteromeric α1β2γ2 GABA channels. Although many types of $GABA_A$ receptors exist, those composed of α1, β2, and γ2 subunits are the most widely expressed in the adult mammalian nervous system, making them the most important for possible cross-over effects. As with the GlyR experiments, oocytes expressing GABA channels were tested for maximal GABA responses, and an EC_{10} GABA concentration was determined. The peptides were then applied using the same pre-application protocol. **Figure 3.2 (A and B)** shows sample tracings of the effects of peptides D12-106 and D12-133 on GABA responses in the a1b2g2 channels. D12-106 produced a minor inhibition of the GABA response, while D12-133 had almost no effect on these channels. The data presented in **Figure 3C** have been scaled to match those presented in **Figure 2C** in order to illustrate that the peptide effects on a1b2g2 $GABA_A$ channels were much smaller than those seen in the GlyRs. As

with the GlyR tests, none of the peptides had any effect in the absence of GABA or altered the holding currents of the oocytes.

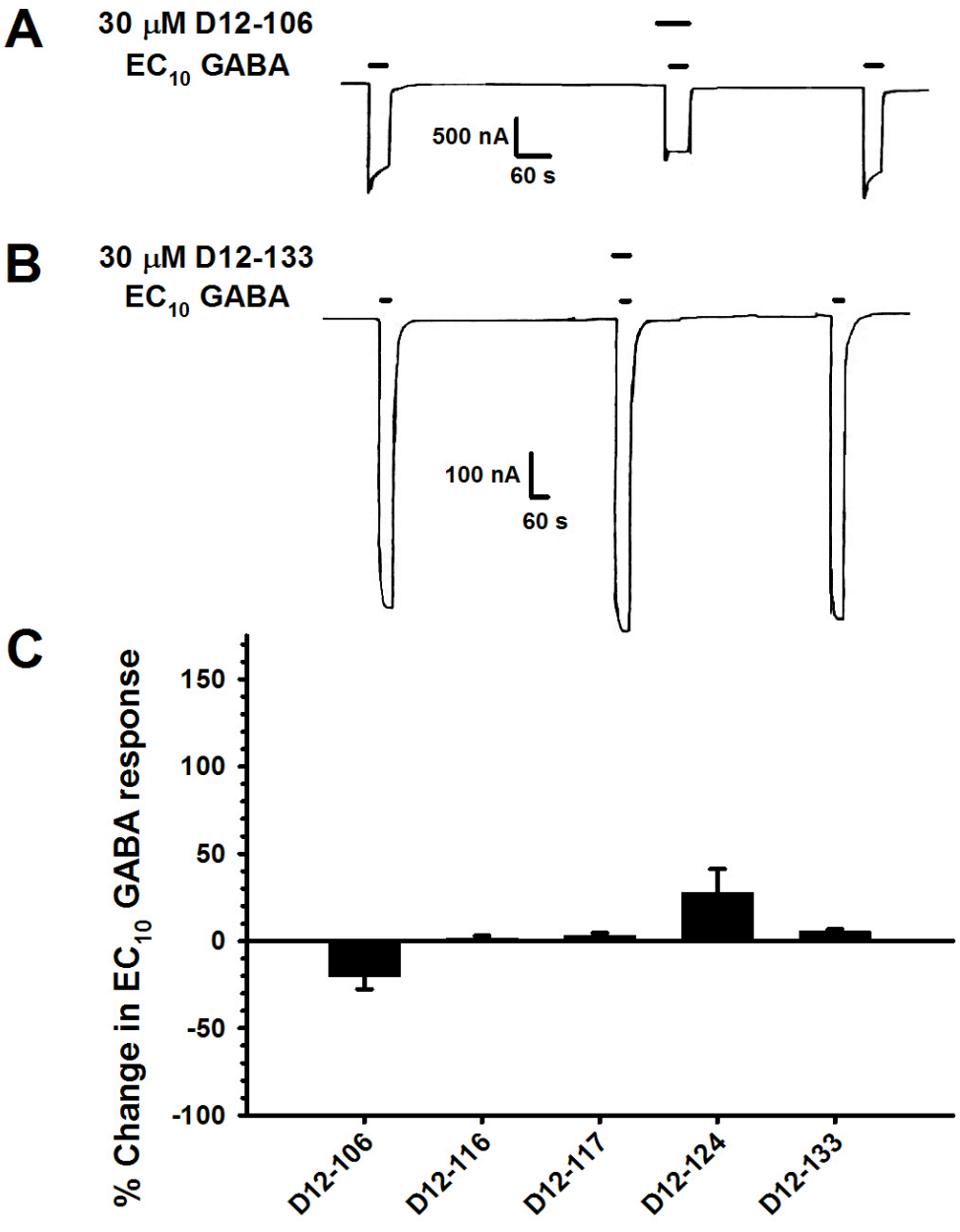

Figure 3.2 – Minimal effects of peptides as allosteric modulators at the $GABA_A$ receptor

(A, B) Representative tracings showing the effects of a 30 μM concentration of peptide D12-106 *(A)* and D12-133 *(B)* on $GABA_A$ α1β2γ2 receptor function. Neither peptide had any effect when applied in the absence of GABA. *(A)* D12-106 showed a small inhibition of the GABA effect when co-applied with GABA following a 30 s application of peptide alone. *(B)* D12-133 had no effect when co-applied with GABA or when applied alone for 30 s. *(C)* Percent changes in responses to EC_{10} GABA produced by 30 μM concentrations of D12 peptides pre-applied for 30 s, followed by co-application with GABA for 30 s. None of the peptides acted as agonists by directly activating the receptor and had minimal to no effects in the presence of EC10 GABA. Data are expressed as the mean ± S.E.

3.3.4: Specificity of Peptide D12-116

Peptide D12-116 produced a marked enhancement of GlyR function ($t = 5.783$, $p < 0.001$) but did not affect $GABA_{A/C}$ responses ($GABA_A$: $t = -0.862$, $p = 0.428$; GABA r1: $t=1.00$, $p=0.391$). This peptide was next assayed with a more stringent specificity test using GABA ρ1 homomeric receptors. **Figure 3.3 (A-D)** compares the effect of this peptide at 30 μM on the EC_{10} responses of both GABA channels. Even at 100 μM of peptide, D12-116 showed no effects on either GABA channel, implying that this effect is due to true specificity, rather than to potential differences in peptide potency among the receptor types. This lack of effect on the channels most structurally similar to the GlyR makes it unlikely that this peptide would have non-specific effects on other, more distantly related channels.

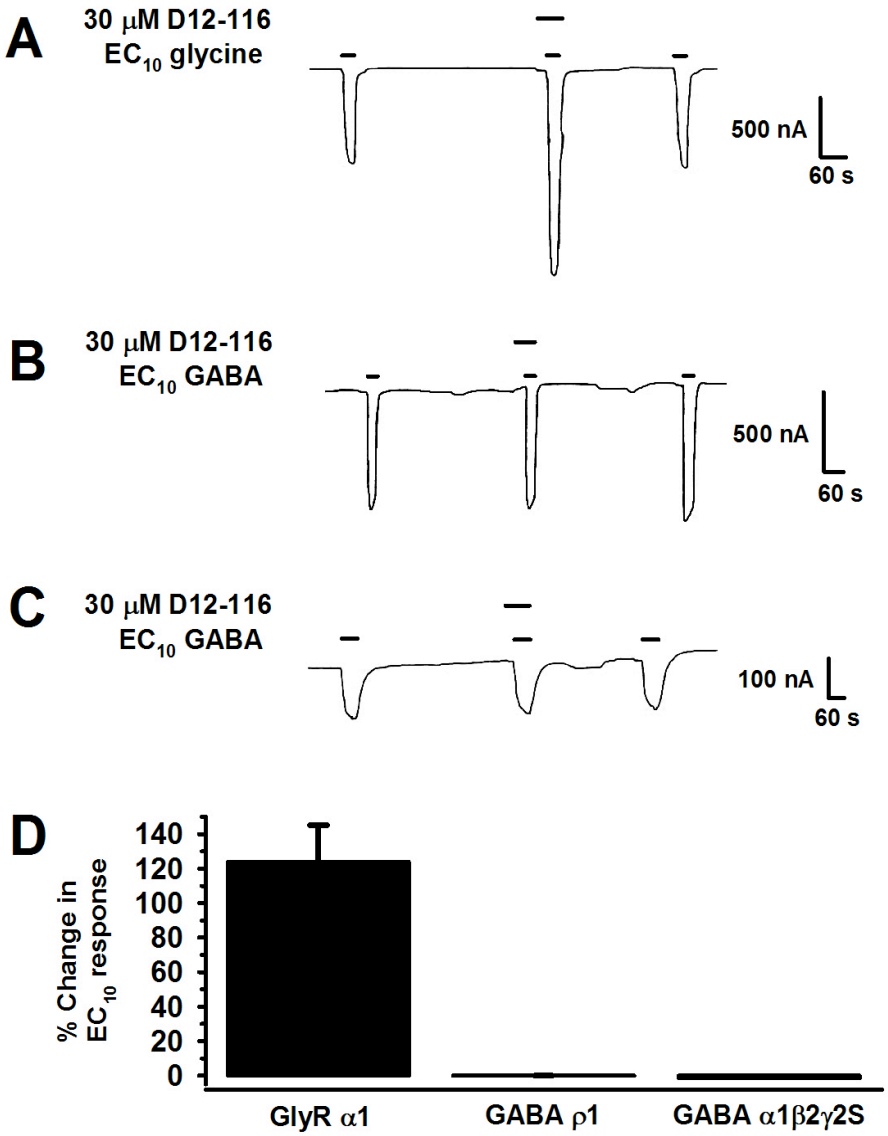

Figure 3.3 – Peptide D12-116 demonstrates specificity for the GlyR

(A - C) Representative tracings of the effect of 30 μM D12-116 on the response elicited by EC_{10} glycine on α1 GlyR *(A)* and EC_{10} GABA on $GABA_A$ α1β2γ2 *(B)* and GABA ρ1 *(C)* receptors. *(A)* D12-116 produced significant enhancement of GlyR α1 function when co-applied with glycine following a 30 s application of peptide alone. The same concentration of peptide had no effect on the function of either $GABA_A$ α1β2γ2 *(B)* or GABA ρ1 *(C)* receptors when co-applied with GABA following a 30 s application of peptide alone. *(D)* Bar graph summarizing the effect of 30 μM D12-116 or the GlyR and two related GABA receptors. D12-116 robustly potentiated the glycine response, but had no effects on either GABA receptor. A one-way analysis of variance showed a significant difference in peptide responses among receptors [$F(2, 16) = 17.9$, $p < 0.001$]. Data are expressed as the mean ± S.E.

3.3.5: Characterization of Peptide D12-116

A concentration-response curve was generated using the D12-116 peptide (**Figure 3.4A**). When co-applied with EC_{10} glycine, the threshold concentration required to elicit the enhancing effect of D12-116 was ~3 μM, and the maximal response occurred at 40 μM peptide. Higher concentrations were less efficacious, resulting in a bell-shaped curve. This could imply that this peptide forms aggregates at higher concentrations or that multiple binding sites with different affinities for the peptide exist on the GlyR. Next glycine concentration-response curves were plotted in the presence and absence of 30 μM D12-116. The peptide significantly left-shifted the glycine curve [$F(1,63) = 130$, $p < 0.001$] without affecting the response to saturating glycine (**Figure 3.4B**). This result suggests that this peptide acts as a positive allosteric modulator of the GlyR, much like alcohols and volatile anesthetics, which also left-shift the glycine concentration-response curve (Mascia et al., 1996a; Krasowski and Harrison, 2000).

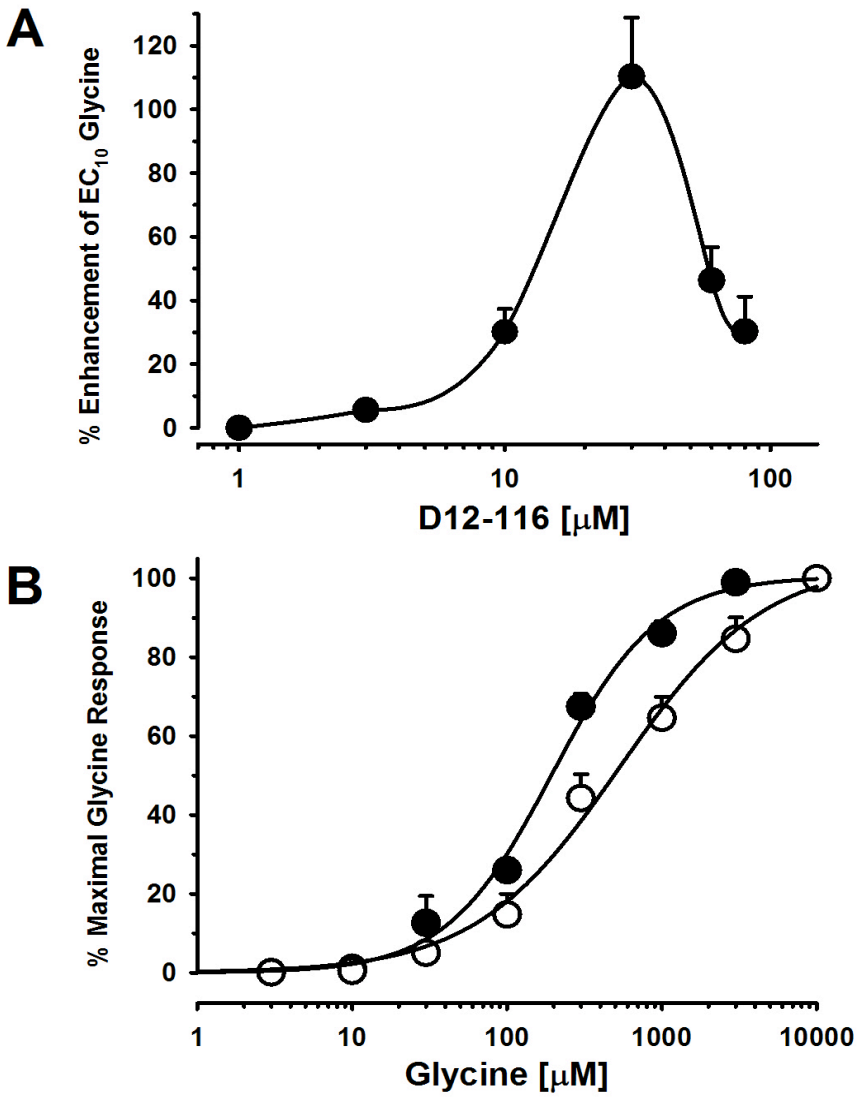

Figure 3.4 – Characterization curves of peptide D12-116

(A) Percent change in the response elicited by EC_{10} glycine co-applied with various concentrations of D12-116 peptide. Oocytes were incubated with 1 – 100 μM of D12-116 for 30 s followed by a co-application of peptide with glycine. None of the peptide concentrations tested elicited currents in the absence of glycine. The threshold peptide concentration for GlyR enhancement is between 3 and 10 μM. *(B)* D12-116 acts as an allosteric modulator of GlyR function by shifting the glycine concentration-response curve to the left. Concentrations of glycine (1 – 10,000 μM) were co-applied with 30 μM D12-116. (○) glycine applied alone. (●) glycine plus 30 μM D12-116. Curves were fit using a four-parameter logistic equation. The EC_{50} of the glycine curve was 796 μM, which was decreased to 196 μM in the presence of peptide. Two-way analysis of variance showed significant effects of glycine concentration [$F(7,63) = 505$, $p < 0.001$] as well as the presence of peptide [$F(1,63) = 130$, $p < 0.001$]. Data are expressed as means ± S.E.

3.3.6: Alanine Scan of Peptide D12-116

When investigating protein-protein interactions, an alanine scan is often used to identify important residues (Wells, 1991; Cunningham and Wells, 1989). In this approach, each amino acid is individually replaced with an alanine residue, which is small and uncharged. Substitution with an alanine removes all side chain atoms past the b-carbon, thus individual alanine mutations can be used to infer the roles of individual side chains. Eleven mutants of D12-116 in which a single amino acid had been replaced with an alanine residue (D12-116 1A-12A) were generated. Residue 9, which is an alanine in the original sequence, was not mutated for this screen. While the majority of these modified peptides did not show significantly different effects from the original D12-116 **(Figure 3.5)**, two mutations caused significant changes in the response to 30 μM peptide. Replacement of the glutamine residue at position 12 significantly reduced the effect of this peptide ($t=2.19$, $p < 0.05$). In addition, the replacement of the isoleucine residue at position 6 significantly increased the peptide effect at 30 μM ($t=3.129$, $p < 0.005$).

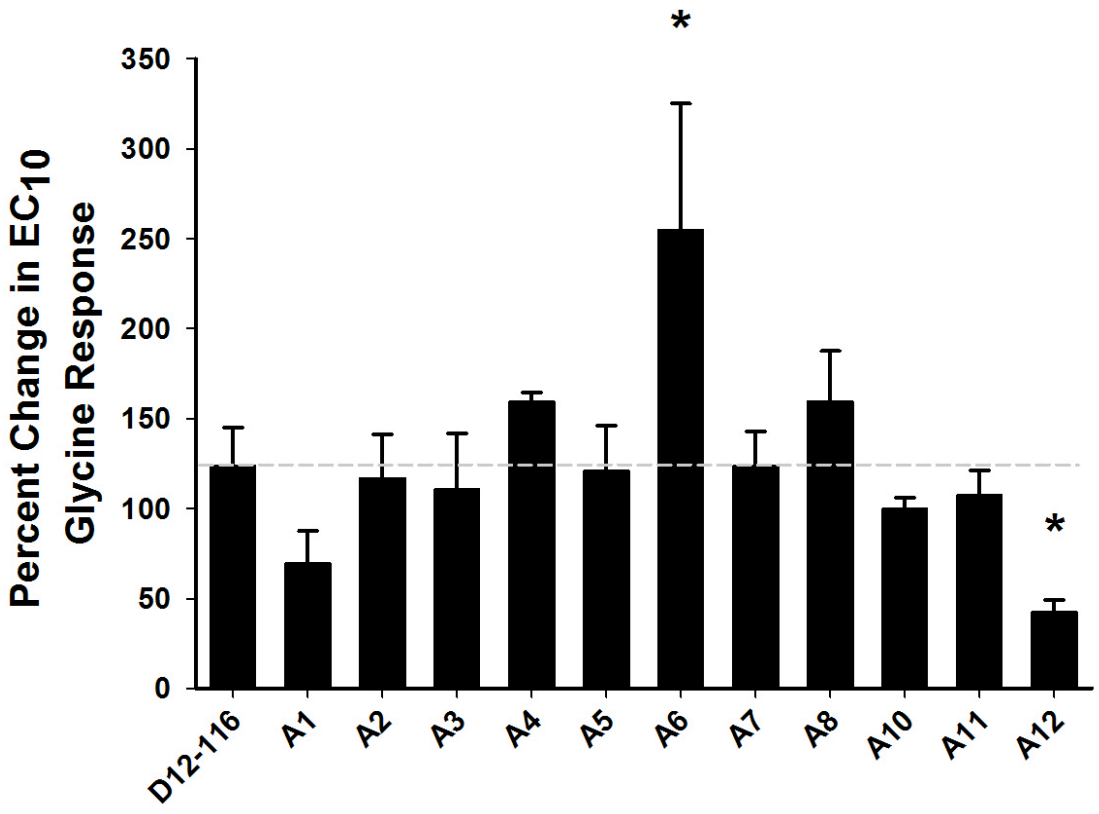

Figure 3.5 – Alanine scan of peptide D12-116 (Y E S I R I G V A P S Q)

Illustration of percent changes in the EC_{10} glycine responses when single amino acid alanine mutants of peptide D12-116 were co-applied with 30 μM peptide. Each residue along the D12-116 peptide was individually replaced with an alanine residue with the exception of residue 9, which is an alanine in the original sequence. The grey line marks the mean peptide enhancement of the original D12-116 peptide (the left-most bar). The degree of enhancement of the EC_{10} glycine response was significantly increased for peptide 116-A6 (YESIR**A**GVAPSQ; $p < 0.005$) and was significantly reduced for peptide 116-A12 (YESIRIGVAPS**A**; $p < 0.05$). Data are presented as mean ± S.E.

3.3.7: Effect of Peptide D12-116 on Different GlyR Subtypes

In mammalian systems, the α1, α2, and α3 GlyR subunits are expressed throughout the CNS (Lynch, 2009). These subunits have shown differential effects to several GlyR modulators, including ethanol. Thus, we compared the effects of 30 μM D12-116 on homomeric α1, α2, and α3 GlyRs. While this peptide robustly potentiates α1 receptors, α2 receptors showed only a weak potentiating response and α3 receptors were inhibited (**Figure 3.6**). GlyRs containing α2 subunits have reduced responses to the enhancing effects of several modulators (Lynch, 2009), thus the reduced response to 116 is unsurprising. However, the inhibitory response of the α3 homomeric channels is striking. The α1 and α3 subunits are more similar in sequence than the α1 and α2 subunits, particularly in the extracellular domain. These data reinforce the idea that these channel subtypes, though highly homologous, may play very different roles *in vivo*.

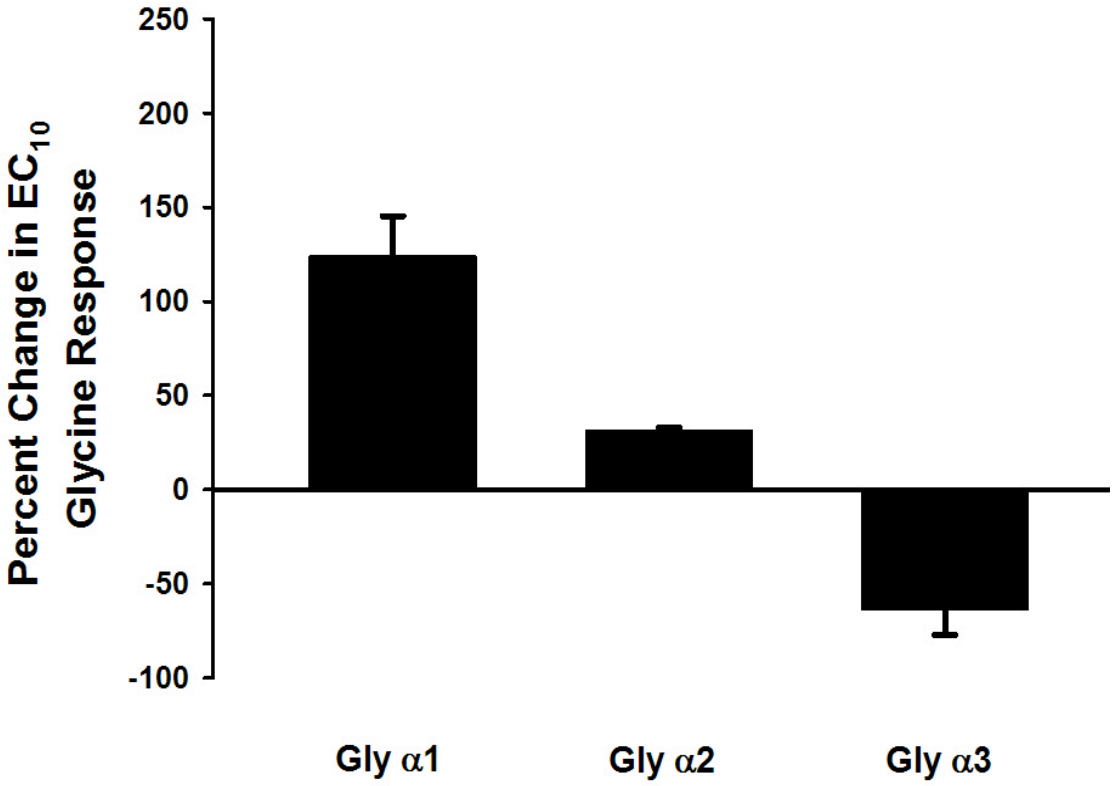

Figure 3.6 – Peptide D12-116 has differential effects on GlyR α subtypes

Percent change in EC_{10} glycine response observed after co-application of glycine with D12-116 at GlyR α1, α2 and α3 homomeric receptors. The peptide robustly potentiated the glycine effect on Gly α1 receptors. The potentiating effect on Gly α2 receptors was reduced compared to α1 receptors. On Gly α3 receptors the peptide had an inhibitory effect. Data are presented as mean ± S.E.

3.4: Discussion

One of the most pharmacologically relevant roles proposed for the GlyR *in vivo* is the mediation of some alcohol-induced behaviors. Many cell-surface proteins, including voltage- and ligand gated ion channels, enzymes, and transporters, are thought to play a role in the effects of ethanol. However, the relative importance of each individual putative target to the effects of alcohol *in vivo* remains poorly understood (Vengeliene et al., 2008). This deficit has been a major impediment to the rational identification of compounds to treat alcohol abuse. The enhancement of inhibitory GlyRs by ethanol is consistent with some of the behavioral consequences of alcohol consumption (Molander and Soderpalm, 2005; Ye et al., 2009). However, because ethanol clearly affects multiple biochemical targets in addition to the GlyR, it has proved difficult to determine conclusively the roles that individual putative targets play in the various behavioral effects of this agent. There would thus be great utility in identifying compounds that can act as either ethanol mimics or ethanol antagonists at only one putative alcohol target. For example, if we were to administer peptide D12-116, which enhanced GlyR function similar to ethanol, intrathecally to rats and observe ataxia, it would suggest that the ataxia produced by ethanol is also mediated by the enhancement of GlyR function.

Although studies using knock-out and knock-in mice (Crabbe et al., 2006) have provided some successes with regard to isolating the effects of individual ethanol targets, these methods, due to their nature, involve a departure from the wild-type phenotype. An alternative approach is the highly specific pharmacological manipulation of individual targets. Allosteric modulation offers the opportunity for subtle manipulations of a target,

rather than complete removal or blockade. Our identification of a GlyR-specific enhancing peptide allows for the emulation of the effects of ethanol at just this one site. The identification of agents that modulate or mimic the actions of ethanol or volatile anesthetics at targets with high specificity could allow for the determination of the contributions of those sites to the overall effects of these compounds without altering the wild-type baseline GlyR function. A better understanding of the relative importance of individual targets could also lead to the development of more targeted therapeutics.

There are many instances of small molecules acting as specific allosteric modulators of proteins (Berg, 2003; May et al., 2004). For example, benzodiazepines bind at nanomolar concentrations to $GABA_A$ receptors composed of specific subunits. They can act as agonists, inverse agonists, or antagonists at a site distinct from the GABA-binding site. These compounds modulate the GABA-mediated currents, but have no effects when applied alone (Mihic et al., 1994). In addition, a number of small molecules, peptides, and modified peptides are known to have substantial effects on neurophysiology, such as enkephalins and conotoxins. Peptides that modulate the function of putative ethanol targets have also been identified (Wilkemeyer et al., 2002; Guzman et al., 2009). For example, the octapeptide NAPVSIPQ antagonizes ethanol inhibition of L1-mediated cell adhesion and is protective against ethanol-induced embryotoxicity (Wilkemeyer et al., 2003).

Previous work used phage display to identify novel peptides that demonstrated high affinities for selected ion channels and acted as either agonists or antagonists at their intended targets (Doorbar and Winter, 1994; Goodson et al., 1994). Peptide libraries

have also been used to identify an antagonist of the *N*-methyl-D-aspartate receptor (Li et al., 1996). In recent years, there has been increasing interest in the development of novel therapeutics that act at allosteric sites rather than acting as agonists or antagonists at the orthosteric ligand-binding site. The hypothesis driving these experiments was that commercially available phage display technology could be used to identify specific allosteric modulators acting to either enhance or inhibit GlyR function, i.e., compounds that left- or right-shift glycine concentration-response curves while having no effects on their own. The peptides we identified acted as allosteric agonists to enhance glycine effects (D12-116) or as inverse agonists that reduced glycine effects (D12-106).

Our approach utilizes the cellular expression of the target protein rather than scaffolding the purified target. This allows for selection in the native environment of the target and minimizes concerns about misfolding of the target protein. As a result, our peptides have the opportunity to bind only in the extracellular region of the channel. Because none of the peptides tested thus far acted as agonists when applied alone, peptides of this size may not be able to bind at the intersubunit orthosteric glycine-binding site (Lynch, 2004) to directly activate the GlyR. In the positive selection portion of the phage display screen, we expressed the GlyR in HEK 293 cells in the absence of exogenously added glycine, so we assume that selection occurred against GlyRs in the closed state. However, glycine is a component in the media in which the HEK cells were grown. While this media was washed away prior to the selection process, it is important to note that the channels used in the selection process are not glycine naïve. To

circumvent possible concerns about cell type-specific binding, we used receptors expressed in *Xenopus* oocytes for the functional tests.

The diversity of peptides identified in our panning screen suggests that a more stringent negative control during phage panning might increase peptide specificity. For example, expressing one or both of the $GABA_{A/C}$ receptor subtypes tested here in the negative selection portion of the panning procedure would be expected to remove those peptides that bind to both GABA and GlyRs, such as D12-106 and D12-124. Another possibility would be to identify peptides that bind only to specific GlyR subunits by expressing GlyR α2 and/or α3 subunits in the negative selection and α1 subunits in the positive selection. The data for 116 suggests that these receptor subtypes can be differentially modulated by peptides, thus this approach could be utilized to address the lack of subunit specific GlyR modulators.

While a high number of isolated peptide sequences with very little sequence homology often implies that the selection process was unsuccessful, each of the ten peptides tested for function modulated the GlyR to some degree (**Figure 3.1**). In addition to validating the selection process, this also suggests that the GlyR contains several potential binding sites in the ECD. Identifying the binding sites for these peptides could lead to the discovery of novel "drugable" sites on the GlyR. The specificity of D12-116 implies that at least one of these binding sites may be unique to the GlyR.

The alanine scan data highlights the utility of phage-derived peptides as pharmacological leads. That is, the original peptide can be systematically adjusted to improve the desired functional effect, leading to the generation of more potent peptides.

Mutation A6, in which the isoleucine at position 6 is replaced with an alanine, significantly enhanced the effect of this peptide. If this version of the peptide maintains specificity for the GlyR, it may be a more useful tool for GlyR characterization than the original 116. This approach also provides information regarding the characteristics of the peptide that are critical for the functional effects. For example, replacing the glutamine at position 12 with a non-polar alanine residue significantly reduced the potentiating effect of this peptide. The location of this residue makes it unlikely that this loss of effect is due to a major change in the folding of the peptide, suggesting instead that the glutamine is important for the interaction of the peptide with the GlyR. In addition to providing important functional information regarding the peptide:channel interaction, the alanine scan also revealed residues that are important or other peptide features, such as solubility. The removal of the proline residue at position 10 greatly decreased the solubility of the peptide in MBS. The stock solution for this peptide was kept at 50 μM because the peptide precipitated out of solution at higher concentrations.

In summary, by pairing a high throughput peptide screen with a standard electrophysiological test, we identified and characterized novel specific peptide allosteric modulators of the GlyR. Other approaches to rapidly identify channel modulators have been developed (Gilbert et al., 2009); however, our method is easily transferable to other channel targets and has minimal set-up requirements. This approach could be modified for almost any target expressed in an adhering cell system and is particularly useful for channels or receptors that currently lack specific allosteric modulators.

4.0 | IDENTIFICATION OF PEPTIDES WITH SUBUNIT SPECIFIC EFFECTS

4.1 - Introduction

There are five known GlyR subunits: α1-4 and β. These channels are pentameric in structure and can be formed of either five a subunits (homomeric) or two α and three β subunits (heteromeric) (Lynch, 2004). There is some experimental evidence that different α subunits can form heteromeric receptors; however, the existence of such channels has not been reported *in vivo* (Kuhse et al., 1993). Prenatally, α2 homomeric receptors are the dominant form of GlyR. After birth, there is a developmental shift to α1β receptors that continues until the third postnatal week (Becker et al., 1988).

While GlyRs containing α1 and β subunits are the most abundant in the adult system, recent reports have identified α2- and α3-containing receptors in several regions of the CNS, often with distinct cellular and regional localizations. For example, several groups have reported extrasynaptic α1, α2 and α3 homomeric receptors in various brain regions. These receptors are primarily located on presynaptic cells and are thought to be involved in modulating the release of glutamate, glycine, and GABA (Turecek and Trussell, 2001; Jeong et al., 2003; Ye et al., 2004). Recently, the expression of α3β GlyR receptors has been reported in the hippocampus (Meier et al., 2005) and nociceptive neurons in the laminae I and II of the spinal cord dorsal horn (Harvey et al., 2004), where they play an important role in inflammatory pain responses (Ahmadi et al., 2002).

The different channel subtypes have distinct kinetic properties, including a slower activation for α2 receptors, reduced agonist potency at α3 receptors, and reduced

conductance in β-containing heteromers (Lynch, 2009). In addition, there is some evidence that the expression of these subtypes may be highly localized. For example, in the retina, different a subunits were found to be associated with specific types of cells (Ivanova et al., 2006). This highly defined localization paired with differing sensitivities to pharmacological agents suggests that individual subtypes may be tailored for specific purposes within the CNS.

To date, there is no pharmacological agent that can differentiate between the different α subunits. Although many compounds exhibit modest subunit-specific differences, none of them can fully discriminate between α1-, α2-, and α3-containing subunits in either homomeric or heteromeric channels. Those compounds that do show some discrimination among the channel types, such as picrotoxin, often have potent effects on other receptor families, which limits their utility (Lynch, 2009). Subtype specific compounds would be useful for differentiating between subtypes *in vitro* and could lead to the development of novel treatments for inflammatory pain and movement disorders. The subunit differences exhibited by the D12-116 peptide (**Figure 3.6**) led to the hypothesis that phage display could be used to identify peptides that discriminate between different α subunits. The panning procedure was modified to include GlyR α2β and α3β channels in the negative selection in order to identify peptides that specifically modulate α1β receptors. This approach yielded several promising peptides, suggesting that this method can be used to identify peptides that discriminate between highly homologous targets.

4.2 - Materials and Methods

Phage selection, site directed mutagenesis and the isolation, injection, and two-electrode voltage clamp of *Xenopus* oocytes were conducted as described in Chapter 2. Specific methods pertaining to the experiments in this chapter are outlined below. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Human Embryonic Kidney cells (HEK 293) were grown according to standard procedures (Freshney, 2002). Cells were cultured at 37^{o}C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate, and 10% fetal bovine serum (Invitrogen). Cell lines were split every 5 days with trypsin/EDTA in Hank's balanced salt solution. Cells were transfected with a total of 8 mg cDNA (1:10 α:β) using Lipofectamine2000 reagent (Invitrogen). All cells were incubated for at least 48 h before use in panning.

Panning day 1: A plate of negative selection cells (expressing either a2b or a3b GlyRs) was washed three times with 0.01 M phosphate-buffered saline (PBS) containing 8.2 mM $NaPO_4$, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl with 1.5% bovine serum albumin (BSA) and 0.1% Tween (PBS/BSA + T). An aliquot containing 2 x 10^{11} phage from the D12 phage library (New England Biolabs) was diluted in 1 ml of PBS/BSA -T. Phage were then applied to negative selection cells ($\alpha2\beta$ or $\alpha3\beta$) and rocked gently at room temperature for 30 min. Phage that did not bind in this negative selection procedure were removed from the plate with a pipette, and transferred to the second negative control plate expressing the other negative control. The order in which

the negative control cells were presented was alternated for each selection round. Phage that did not bind in the second negative control were removed using a pipette, applied to a plate of α1β GlyR-expressing cells (positive selection), and rocked gently at room temperature for 60 min. Non-binding phage were discarded, and the plate was washed five times with PBS/BSA-T. Elution of the bound phage was performed by lowering the pH using 0.2 M glycine HCl (10 M HCl buffered to pH 2.2 with glycine) plus 1 mg/ml BSA and rocking the cells at room temperature for 10 min. The eluate was removed and neutralized with 150 ml of 1 M Tris-HCl (pH 9.0). Titering was performed, and the remainder of the eluate was added to 20 ml of *E. coli* (A_{600}) in LB broth. After 4.5 h or incubation, the culture was transferred to a 50-ml Falcon centrifuge tube and spun at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and respun. The upper 80% of the supernatant was again transferred to a fresh tube and a one-sixth volume of polyethylene glycol/NaCl (20% w/v polyethylene glycol 8000 and 2.5 M NaCl) was added. Phage were allowed to precipitate overnight at 4°C.

Panning day two: The polyethylene glycol precipitates were spun at 10,000 rpm for 15 min at 4°C. The supernatant was decanted, and the precipitate was spun again. Residual supernatant was removed using a pipette. The pellet was resuspended in 1 ml of Tris-buffered saline (50 mM Tris-HCl pH 7.5 and 150 mM NaCl), transferred to a 1.7-ml microcentrifuge tube, and spun at 10,000 rpm for 5 min at 4°C. In a fresh tube, the suspended phage were reprecipitated with polyethylene glycol/NaCl on ice for 60 min. After spinning at 10,000 rpm for 10 min at 4°C, the supernatant was discarded. The pellet was resuspended in Tris-buffered saline and spun again for 1 min. The supernatant

was then transferred to a fresh tube and stored at 4°C. Aliquots of the amplified phage were titered at three serial dilutions.

For successive panning rounds, the amplified phage were diluted in PBS/BSA-T so that the input concentration was always 2 x 10^{11} virons. At the end of five rounds of panning, individual plaques from the most recent titer plates were isolated and incubated overnight in LB broth at 37°C with agitation. The overnight culture was then purified using the S.N.A.P. MiniPrep kit (Invitrogen) and the phage DNA was sequenced in-house using a -96gIII sequencing primer (New England Biolabs). Individual peptide sequences were sent to Peptide 2.0 Inc. (Chantilly, VA) for synthesis. Peptides were received as lyophilized powders of TFA salts. The peptides were dissolved in MBS at a concentration of 200 μM and stored at 4°C.

Custom oligonucleotide primers were designed using the NetPrimer primer design program (Premier Biosoft) and obtained from Integrated DNA Technologies (Coralville, IA). The following primers were used to generate the mutations: Group 1 – F: GAG GCT GAA GCT GCT CGC TCC CGA TCC GCG CCT ATG TCA CCC TCG GAT TTC CTG, R: CAG GAA ATC CGA GGG TGA CAT AGG CGC GGA TCG GGA GCG AGC AGC TTC AGC CTC; Group 2 – F: GAC CTC ATC TTT GAG TGG CAG GAT GAG GCA CCC GTG CAG GTA GCA GAT GGA CGA ACT CTG, R – CAG AGT TAG TCC ATC TGC TAC CTG CAC GGG TGC CTC ATC CTG CCA CTC AAA GAT GAG GTC; S121F – F: GAC AAC AAA TTG CTA AGG ATC TTC CGG, R: CCG GAA GAT CCT TAG CCA TTT GTT GTC; A212V – F: GGT AAA TTC ACC TGC ATT GAG GTC CGG TTC CAC CTG, R: CAG GTG GAA CCG GAC CTC AAT

GCA GGT GAA TTT ACC. All mutants were made using the a1 GlyR cDNA as the template and the Stratagene (Cedar Creek, TX) QuickChange Mutagenesis Kit. The forward and reverse primers (125 ng/ml) were individually combined with the template a1 cDNA (25 ng/ml), nucleotide triphosphates, and Taq DNA polymerase and run through the first 15 cycles of a linear amplification thermocycling protocol in a PTC-100 thermocycler. The forward and reverse mixtures were then combined 1:1 and run together for another 15 cycles.

Two high-resistance (0.5 – 10 MW) glass electrodes filled with 3 M KCl were used to impale the animal poles of isolated oocytes injected with
GlyR cDNA (1:20 a:b). Cells were voltage-clamped at -70 mV using a Warner Instruments OC-725C oocyte clamp (Hamden, CT), and MBS was perfused over them at a rate of 2 ml/min using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co, Vernon Hills, IL) through 18-gauge polyethylene tubing. All peptide solutions were prepared in MBS, and all applications (30 sec) were followed by 5 – 10 min washout periods as appropriate.

Current peak responses were measured from chart recorder tracings. Peptide effects were calculated as percent changes compared with the effects produced by glycine in the absence of peptide. In all cases, oocyte data were obtained from at least two different frogs. The percent enhancement or inhibition values obtained in the presence of peptide were compared by either t-test or one- or two-way analysis of variance, as indicated, to determine statistical significance with a criterion of $p < 0.05$.

4.3 - Results

4.3.1: Peptides Isolated from Phage Panning

For these experiments I chose to use αβ heteromeric receptors rather than α homomers, as these are the dominant form found in the adult nervous system. It should be noted that while the α1, α2, and β subunits were cloned from human DNA, the α3 receptors used here were cloned from rat DNA. However, the GlyR family is highly conserved across several mammalian species, and the human α3 subunit only differs from the rat α3 subunit by four amino acids, only one of which is in the extracellular portion of the channel (Hirata et al., 2009). Thus, it is unlikely that the use of rat α3 in the negative selection procedure significantly influenced the affinities of the selected peptides. The Ph.D.-7™ phage display library (New England Biolabs) was used for the selection process. This library is based on a combinatorial library of random heptapeptides fused to the N-terminus of the pIII phage coat protein. According to the manufacturer, this library contains approximately 2.8×10^9 electroporated sequences with approximately 70 copies of each sequence per 10 μl of phage. Following seven rounds of phage selection and amplification, 25 colonies were isolated and sent for sequencing. Similar to the original D12 panning, several different sequences were obtained. No clear regions of homology were identified between the different collected sequences (**Table 4.1**). Given the stringency of the panning, this was somewhat surprising. However, the ECD of these channels is very large, offering several potential binding sites.

Phage	Sequence						
D7.2-012	S	I	R	L	D	S	Q
D7.2-013	H	A	P	K	S	D	T
D7.2-014	D	R	M	P	H	Y	F
D7.2-015	S	T	F	T	K	S	P
D7.2-016	T	G	A	D	L	N	T
D7.2-017	Y	T	M	P	G	E	L
D7.2-018	V	I	P	H	V	L	S
D7.2-019	G	V	Q	I	M	G	R
D7.2-110	N	A	N	A	A	L	P
D7.2-111	S	W	Q	Q	G	P	Y
D7.2-111B	S	T	I	H	G	S	T
D7.2-112	G	S	S	C	C	K	T
D7.2-113	S	I	L	P	Y	P	Y
D7.2-115	K	L	P	G	W	S	G
D7.2-116	N	Q	L	T	T	L	N
D7.2-118	A	A	P	T	V	P	R
D7.2-119	Q	E	T	R	A	P	G
D7.2-120	N	Q	L	P	L	H	A
D7.2-121	G	P	M	L	A	R	G
D7.2-122	T	T	M	P	I	D	S
D7.2-123	T	T	P	T	K	S	A
D7.2-124	V	Q	T	Y	A	R	G
D7.2-125	T	S	L	N	R	Y	P
D7.2-126	S	H	T	A	P	L	R
D7.2-128	D	V	P	V	P	Q	V

Table 4.1: Sequences of peptides identified by panning against α1β GlyR

A total of 25 sequences were collected following seven rounds of panning. Each sequence was seen only once. The diversity of the peptide sequences was high, with no clear regions of homology.

4.3.2: Functional Analysis of Selected Peptides

Two-electrode voltage clamp electrophysiology in *Xenopus* oocytes was used to characterize the functional effects of these peptides on α1β GlyRs. A maximally effective concentration of glycine (10 mM) was first applied to determine the level of GlyR expression. This maximal response was used to determine a low concentration of glycine that elicited a response equal to ~10% of the maximal current (EC_{10}, where EC is the effective concentration). The EC_{10} was applied several times to ensure stable baseline glycine responses. The oocyte was then incubated with 30 μM of a peptide for 30 s, followed by a 30 s co-application of the peptide and EC_{10} glycine. None of the tested peptides had any effect in the absence of glycine. This implies that they neither function as agonists at the glycine-binding site nor do they affect the functioning of any other naturally occurring oocyte proteins that might influence the holding current. A variety of modulatory effects, ranging from 103% potentiation to 25% inhibition, were seen across the different peptides **(Figure 4.1),** confirming that these peptides were selected for their affinity to the GlyR and not as the result of non-specific binding to other cellular targets.

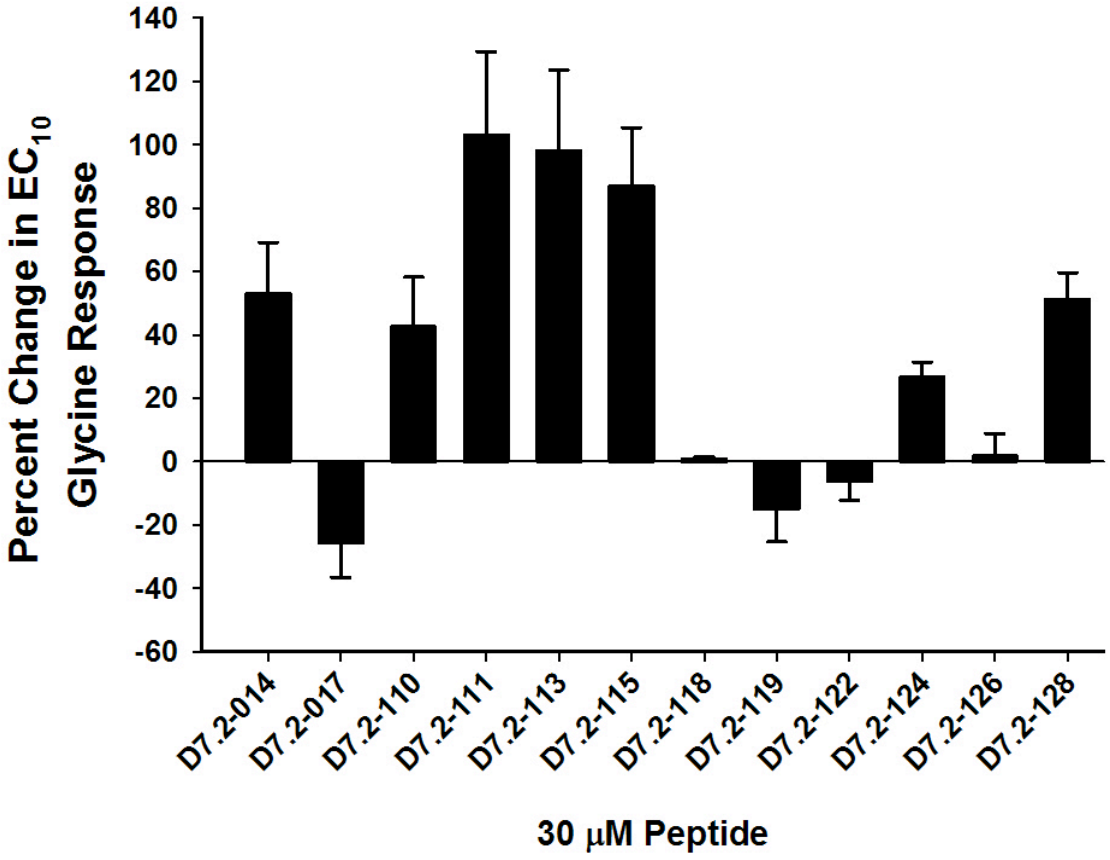

Figure 4.1 – Identification of D7 peptides that allosterically modulate Gly α1β receptor function

Bar graph representing the percent change in the response to EC_{10} glycine when co-applied with 30 μM concentrations of various D7 peptides. None of the peptides showed any effects on holding currents when applied in the absence of glycine. Data are presented as mean ± S.E.

4.3.3: Specificity of Isolated Peptides

The peptides were next tested for their functional effects on the other GlyR α subtypes. I hypothesized that the inclusion of α2β and α3β in the negative selection of the panning procedure would bias the selection towards peptides with affinity for α1-containing receptors. After establishing an EC_{10}, the oocyte was incubated with 30 μM of a peptide for 30 s, followed by co-application of the peptide and EC_{10} glycine. A comparison of the responses to 30 μM peptide on all three receptor subtypes is shown in **Figure 4.2.** Most of the peptides displayed a clear reduction in effect at the α2-containing receptors compared to the α1-containing receptors. The selection against α3 binding was slightly less successful, as most of the isolated peptides modulated α3-containing receptors. However, the α1 and α3 GlyR subunits are 98% homologous in the ECD. Thus, that phase of the phage selection was very stringent, and it is unsurprising that many of the peptides also recognize α3-containing receptors. Three of the tested peptides, D7.2-014, D7.2-111, and D7.2-128 showed significant selectivity for α1β GlyRs over the α2β and α3β receptors [D7.2-014: $F(2,10) = 4.789$, $p < 0.05$; D7.2-111: $F(2,8) = 7.53$, $p < 0.03$; D7.2-128: $F(2,10) = 15.65$, $p < 0.003$].

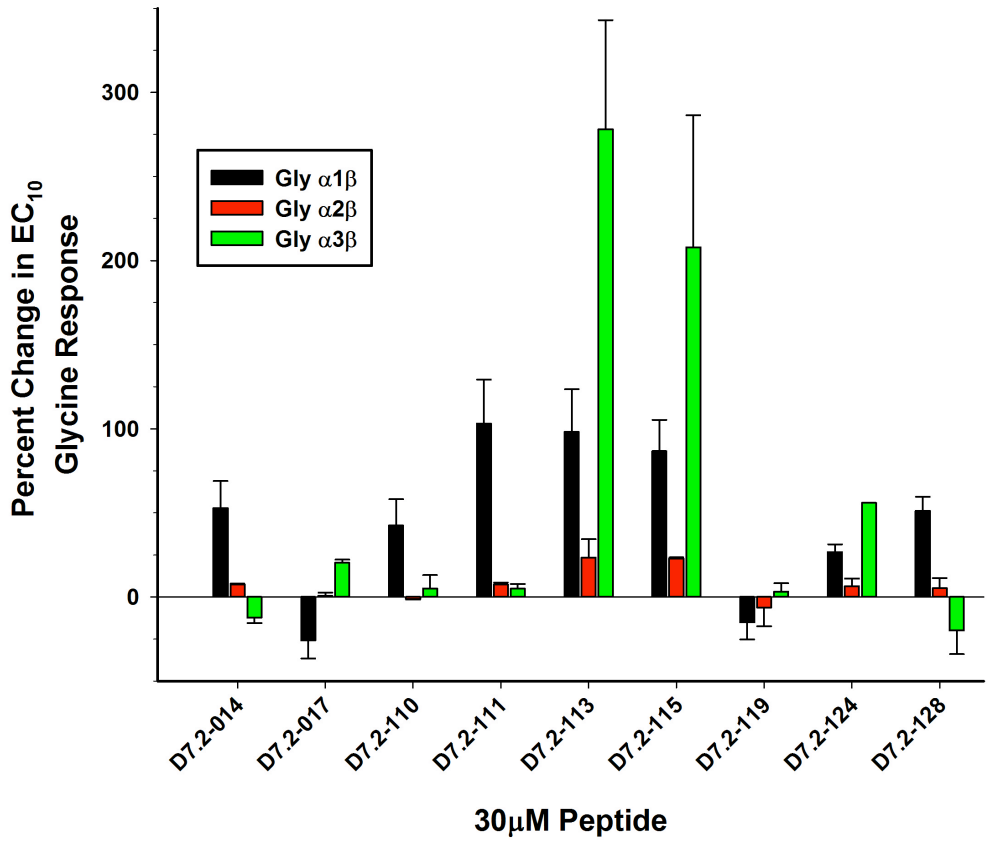

Figure 4.2 – Differential effects of D7 peptides on GlyR subtypes

Effects of 30 μM D7 peptides on the EC_{10} glycine responses of α1β (black bar), α2β (red bar) and α3β (green bar) GlyR. None of the peptides had any effect on any channel subtype when applied in the absence of glycine. As described in detail in the text, the peptides varied in their abilities to distinguish among receptor subtypes. Data are presented as mean ± S.E.

4.3.4: Effects of GlyR α1 Mutations

While several of our peptides show a clear preference for α1-containing receptors over α2- and α3-containing receptors, it is unclear if this difference is due to a reduced affinity for these subtypes or a difference in how the binding signal is integrated by the different channel subtypes. In order to identify extracellular residues that are critical for these peptide differences, we generated α1 GlyR subunits containing mutations at the residues that differ from α3. (**Figure 4.3**). As several of the differences between the subunits occur in clusters, two major group mutations were made. Group 3.1 included three mutations (A4R, P5S, and K6A), and group 3.2 included four mutations (E172D, Q173E, G174A, and A175P). Two single point mutations, S121F and A212V, were also generated. If the difference in peptide effect is due to binding at a non-conserved location, mutating these residues should prevent the peptide effect. The effects of 30 μM D7.2-014 on the EC_{10} glycine response in these mutants were recorded and compared to that of wild-type α1β. While none of the mutations fully abolished the peptide effect, the group 3.1 mutation reduced the peptide effect without altering the sensitivity to glycine (**Figure 4.4**).

GlyRα1:ARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPVNVSCN
GlyRα3:ARSRSAPMSPSDFLDKLMGRTSGYDARIRPNFKGPPVNVTCN

IFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPDDSLDLDPSML
IFINSFGSIAETTMDYRVNIFLRQKWNDPRLAYSEYPDDSLDLDPSML

DSIWKPDLFFANEKGAHFHEITTDNKLLRISRNGNVLYSIRITLTLACP
DSIWKPDLFFANEKGANFHEVTTDNKLLRIFKNGNVLYSIRLTLTLSCP

MDLKNFPMDVQTCIMQLESFGYTMNDLIFEWEQGAVQVADGLTLP
MDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQDEAPVQVAEGLTLP

QFILKEEKDLRYCTKHYNTGKFTCIEARFHLERQ
QFLLKEEKDLRYCTKHYNTGKFTCIEVRFHLERQ

Figure 4.3: Sequence alignment of the ECD of human GlyR α1 and rat GlyR α3

Residues that differ between the hGlyR α1 and rGlyR α3 are highlighted. The mutations generated for these experiments are highlighted in yellow, while all other differences are highlighted in blue. The α3 subunit contains six additional residues at the beginning of the sequence that do not appear in the α1 subunit. For the purposes of this alignment, those residues are not listed. Sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). hGlyR α1: NP_001139512.1 rGlyR α3: NP_446176.3

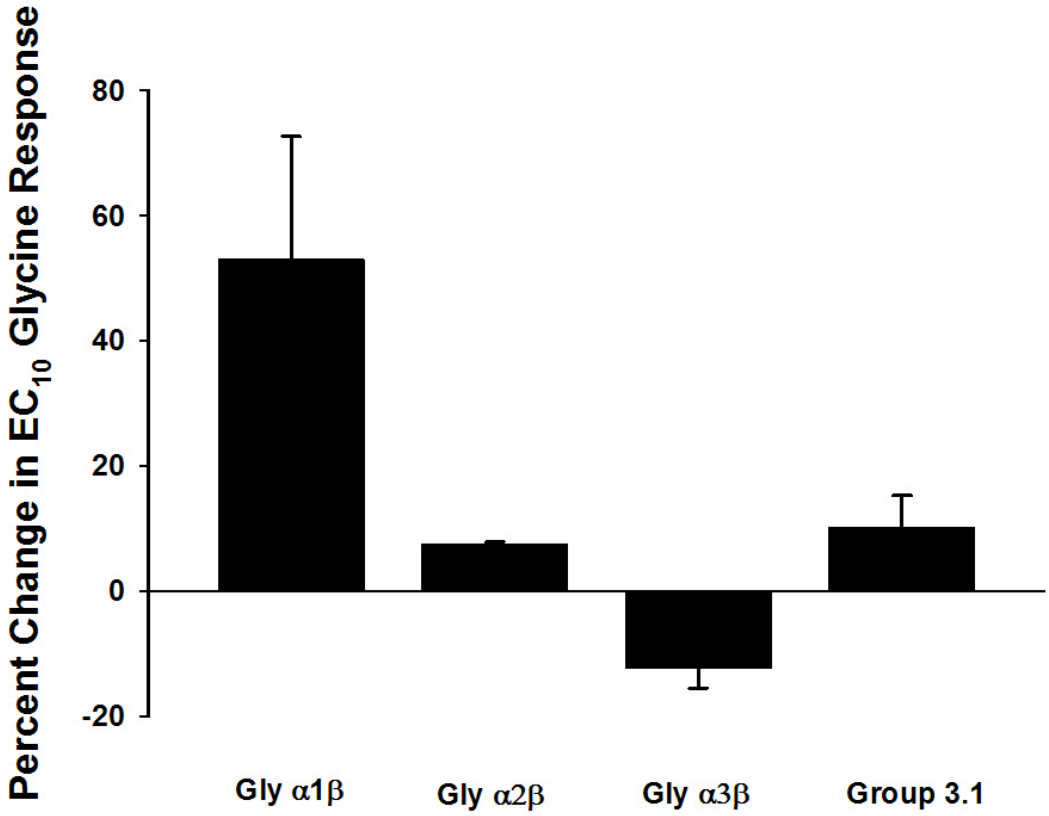

Figure 4.4 – Group mutation 3.1 reduces the effect of peptide D7.2-014

Percent change in EC_{10} glycine response observed after co-applying glycine with 30 μM D7.2-014. D7.2-014 robustly potentiated Gly α1β channels, while showing only a slight potentiation of α2β channels and a slight inhibition of α3β channels. Mutating three residues in the α1 subunit to their counterparts in the α3 subunit (A4R, P5S, K6A; group mutation 3.1) greatly reduced the potentiating effect of D7.2-014. Data are presented as mean ± S.E.

4.3.5: Characterization of Peptides

Concentration response curves were generated for several peptides in order to determine their range of effectiveness. Various peptide concentrations were applied to stable EC_{10} glycine responses in $\alpha1\beta$ GlyRs. Rather than the expected sigmoidal or bell-shaped concentration response curves, most of the peptides exhibited bimodal curves, with a sharp peak in the low micromolar range, followed by a decrease and subsequent reappearance of the effect at higher concentrations. Other peptides had inhibitory effects at low concentrations, but potentiating effects at higher concentrations (**Figure 4.5**). The occurrence of these atypical curves for several peptides across several batches of eggs suggests that these effects are a true phenomena and not simply random occurrences. These results suggest a more complex mechanism of action.

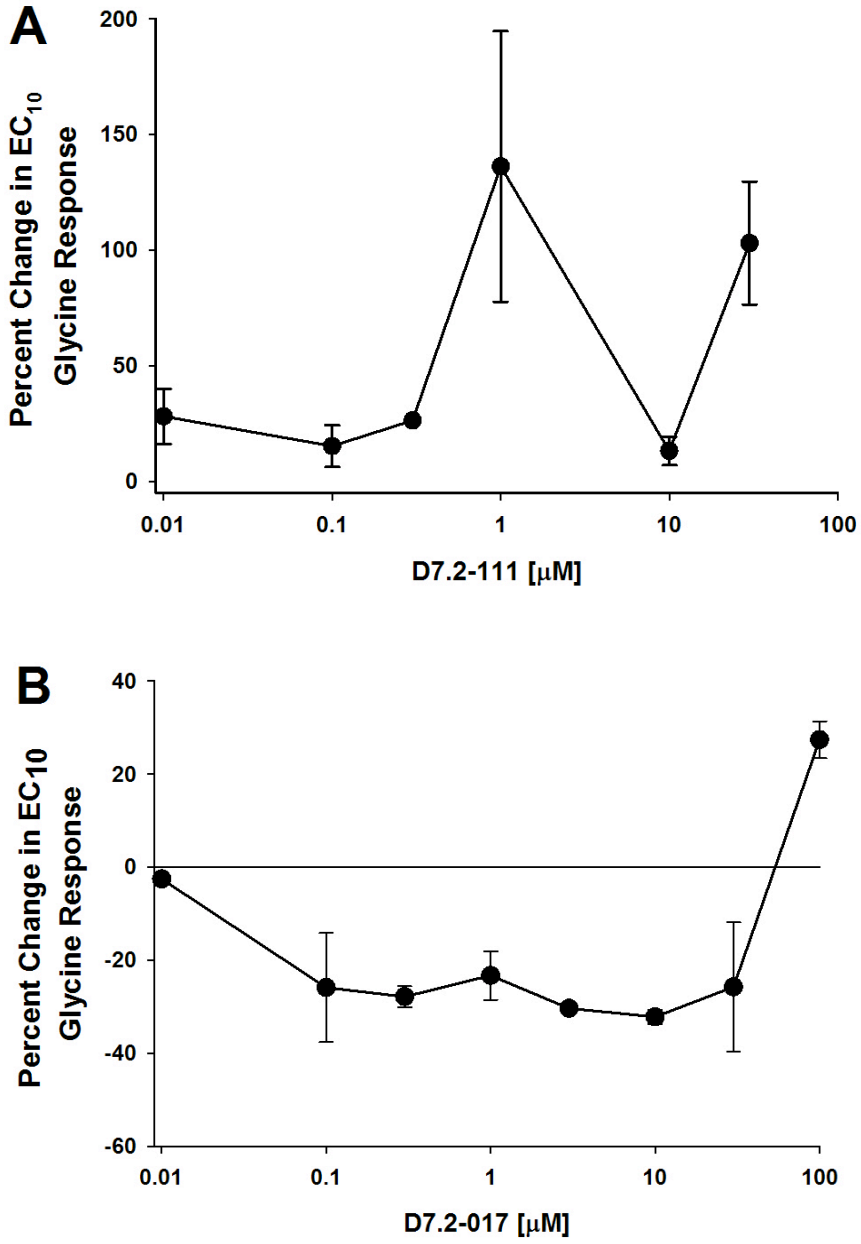

Figure 4.5 – D7 peptides display atypical concentration-response curves

Concentration-response curves obtained from two D7 peptides. *(A)* Percent change in the EC_{10} glycine response when glycine was co-applied with 0.01 – 30 μM of D7.2-111. D7.2-111 shows an enhancing effect of the glycine response at a concentration of 1 μM peptide and this quickly drops off at 10 μM, followed by a rebound in effect at 30 μM. *(B)* Percent change in EC_{10} glycine response when glycine was co-applied with 0.01 – 100 μM of D7.2-017. This peptide was inhibitory at low concentrations, but then produced a potentiating effect above 30 μM. Data are presented as mean ± S.E.

4.4 - Discussion

The discovery of α2- and α3-containing GlyRs throughout the CNS has prompted a shift in the general assumptions about the roles of the various GlyR subtypes. Although the four known α subtypes share high sequence homology, they have differing pharmacological properties and CNS distributions, which could have significant implications for GlyR-influenced behaviors. The idea that channels composed of differing subtypes could have significantly different roles in the CNS is in line with data from other Cys-loop receptors. For example, $GABA_A$ receptors, the other anionic member of the Cys-loop family, have widely varying pharmacological properties and suggested roles depending on their subunit composition. Within this family, $GABA_A$Rs containing α2 subunits are responsible for the anxiolytic effects of benzodiazepines, while α1-containing $GABA_A$Rs mediate the sedating properties of these drugs (Atack, 2010).

The growing interest in GlyR α subtype differences has highlighted an important gap in GlyR pharmacology. None of the known GlyR modulators are capable of distinguishing between α1-, α2, and α3-containing receptors. While some modulators are more or less potent at a given subtype, none of them are fully specific, limiting their use in experimental studies aimed at isolating the contributions of one subtype (Lynch, 2009). In an effort to address this lack of subtype-specific modulators, I adapted my phage display protocol to identify peptides that show subunit specificity (Tipps et al., 2010). These experiments were designed to test the hypothesis that the inclusion of α2β

and α3β receptors in the negative selection process would bias the selection toward peptides that show preferential affinity for α1β receptors.

As with the previous selection, a diverse pool of peptides was identified. This diversity was somewhat surprising given the stringency of the panning process. The a subunits are >90% identical in the extracellular regions, thus including α2- and α3-containing receptors in the negative selection significantly reduces the quantity of "unique" binding sites in the positive selection. However, amino acid sequence homology does not necessarily translate into identical secondary structures, thus even regions that share amino acid sequences between the channel subtypes may offer binding sites that are not structurally identical due to differences.

While the selectivity for α1β over α2β GlyR was highly successful, only a few peptides were able to discriminate between α1β and α3β receptors. At 30 μM, D7.2-128 significantly enhanced the response of α1β GlyR ($t = 6.022$, $p < 0.005$), without significantly altering the responses of α2β or α3β GlyRs (α2β: $p = 0.046$, α3β: $p = 0.392$). However, this specificity did not hold at higher concentrations. Given the high sequence homology of α1- and α3-containing GlyRs, it is not surprising that many peptides would display affinity for both subtypes. During the panning process, phage carrying peptides with high affinity for α2β and α3β GlyRs would be largely selected out; however, peptides with a low affinity for these channels and a high affinity for α1β would likely not be fully removed and would thus be carried on to subsequent rounds. Adjustments in the selection process, such as increased rounds of negative selection prior to the positive selection, may help to resolve this problem.

There are several possible explanations for the atypical concentration-response curves observed. One possibility is that multiple binding sites with differing affinities exist on the GlyR, and thus the observed effects are actually a combination of two distinct actions. This could explain why none of the mutations fully inhibited the peptide effect. However, without any indication of where these peptides may be binding, it is difficult to address the question of specific binding sites. It is also possible that aggregation, a common complication when dealing with peptides, occurs at higher peptide concentrations (Chi et al., 2003). A reduction in free peptide due to aggregation followed by a slow reaccumulation could explain the drop and subsequent reinstatement of the peptide effect. While it is extremely likely that aggregation occurs for a subset of the tested peptides, this phenomenon is difficult to measure for small peptides, especially when the aggregation occurs at low concentrations. A third option is the presence of a contaminant that also modulates the GlyR. This possibility will be explored in the following chapter.

In conclusion, while none of the peptides identified in this panning showed complete specificity between the three α subtypes, several peptides showed preferential modulation of $\alpha1\beta$ GlyR over $\alpha2\beta$ and $\alpha3\beta$ GlyRs. These peptides are promising leads toward the development of more specific compounds. The effect of D7.2-014 was altered by mutating residues within the $\alpha1$ subunit, suggesting that the differential peptide effects across the GlyR subtypes are mediated by specific amino acids.

5.0 | TRIFLUOROACETATE MODULATES THE GLYCINE RECEPTOR

5.1 - Introduction

Trifluoroacetic acid (TFA) is a strong carboxylic acid. In peptide synthesis, it is used as an ion-pairing agent in high-performance liquid chromatography (HPLC) purification. The addition of TFA increases the hydrophobicity of molecules by forming ionic pairs with their charged groups. This allows for interactions between the molecules and the hydrophobic stationary phase, thus enabling separation (Garcia, 2005). TFA binds to the free NH_2 termini of peptides and the side chains of lysine, histidine, and arginine residues (Cornish et al., 1999). The ion pairs formed by TFA are extremely strong, and cannot be removed without an additional ion replacement step during purification. As a result, HPLC prepared peptides often exist as trifluroacetate salts. The presence of TFA can alter the behavior of a peptide or modify its conformation (Roux et al., 2008).

In its acetate form, TFA affects several biological processes. The most common source of free TFA in the body is the metabolism of the volatile anesthetics halothane, isoflurane and desflurane (Cohen, 1971; Hitt et al., 1974). It has been suggested that TFA as a metabolic end product may be responsible for the development of halothane-induced hepatitis and neurotoxicity (Gut et al., 1995; Ma et al., 1990), as well as playing a role in the cardioprotective effects of isofluorane (Han et al., 2001). In addition, TFA alters the proliferation rates of certain cell types, increasing the growth rate of some, while reducing the growth of others (Cornish et al., 1999). The exact mechanisms

underlying these effects on cell reproduction are unknown; however, TFA is a chaotropic anion, and other compounds of this class can have an impact on membrane function, enzymatic catalysis, secondary protein structure, and protein stability.

All of the peptides investigated in this thesis were tested as TFA salts. In an attempt to address some of the inconsistencies in the peptide modulation of the glycine receptor (GlyR), we examined the effects of TFA on GlyR function. We found that TFA is not only able to reversibly modulate glycine responses, but also shows differential modulation of receptor subtypes. This is important both with regard to the testing of future peptides and possible secondary effects following the administration and metabolism of volatile anesthetics, for which the GlyR is a major target (Harris et al., 1995).

5.2 - Materials and Methods

The isolation, injection and two-electrode voltage clamp of *Xenopus* oocytes were conducted as described in Chapter 2. Specific methods pertaining to the experiments presented in this chapter are outlined below. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Two high-resistance (0.5 – 10 MW) glass electrodes filled with 3 M KCl were used to impale the animal poles of isolated oocytes injected with
GlyR cDNA (1:20 a:b). Cells were voltage-clamped at -70 mV using a Warner Instruments OC-725C oocyte clamp (Hamden, CT), and MBS was perfused over them at a rate of 2 ml/min using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co,

Vernon Hills, IL) through 18-gauge polyethylene tubing. All peptide and chemical solutions were prepared in MBS. All applications (15 - 30 sec) were followed by 5 – 10 min washout periods as appropriate.

Current peak responses were measured from chart recorder tracings or calculated from digital LabChart files. Peptide effects were calculated as percent changes compared with the effects produced by glycine in the absence of peptide. The percent enhancement or inhibition values obtained in the presence of peptide were compared by either one- or two-way analysis of variance, as indicated, to determine statistical significance, with a criterion of $p < 0.05$.

5.3 - Results

5.3.1: TFA Modulates α1β GlyRs

Low concentrations of glycine generating a response corresponding to 10% of the maximal response (EC_{10}) were applied alone and in the presence of various concentrations of TFA (**Figure 5.1**). TFA reversibly enhanced the glycine response in a concentration-dependent manner. The potentiating effect began around 0.0005 mM, reaching a peak at 0.01 mM**.** Preincubation of the oocyte with TFA alone did not alter the holding current, suggesting that TFA cannot activate the channel and is not altering the function of any of the other endogenous oocyte proteins responsible for the holding current. In addition, TFA did not alter the response elicited by a maximally effective concentration of glycine, suggesting that it acts as an allosteric modulator of the GlyR (**Figure 5.2**). Because TFA has been shown to alter membrane function, we tested the

effect of TFA alone on the holding currents generated by a range of command voltages (0 to -70 mV) to rule out non-GlyR effects on the oocyte membrane. None of the concentrations tested (0.001 mM – 10mM) had any effect on the holding current at any of these membrane potentials.

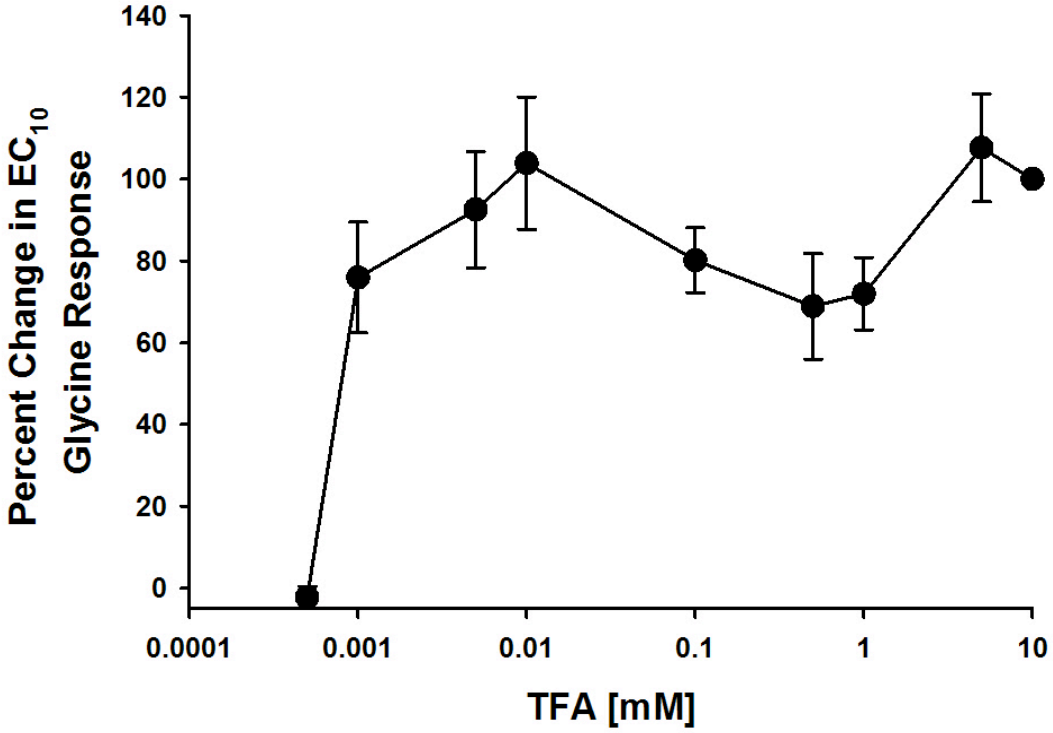

Figure 5.1 – Trifluoroacetate (TFA) potentiates α1β GlyR function

EC_{10} glycine was co-applied with various concentrations of TFA (0.0005 – 10 mM). A robust potentiating effect was seen at 0.001 mM TFA and appeared to plateau by 0.01 mM. None of the concentrations of TFA tested had any effects on holding currents when applied in the absence of glycine. Data are presented as mean ± S.E.

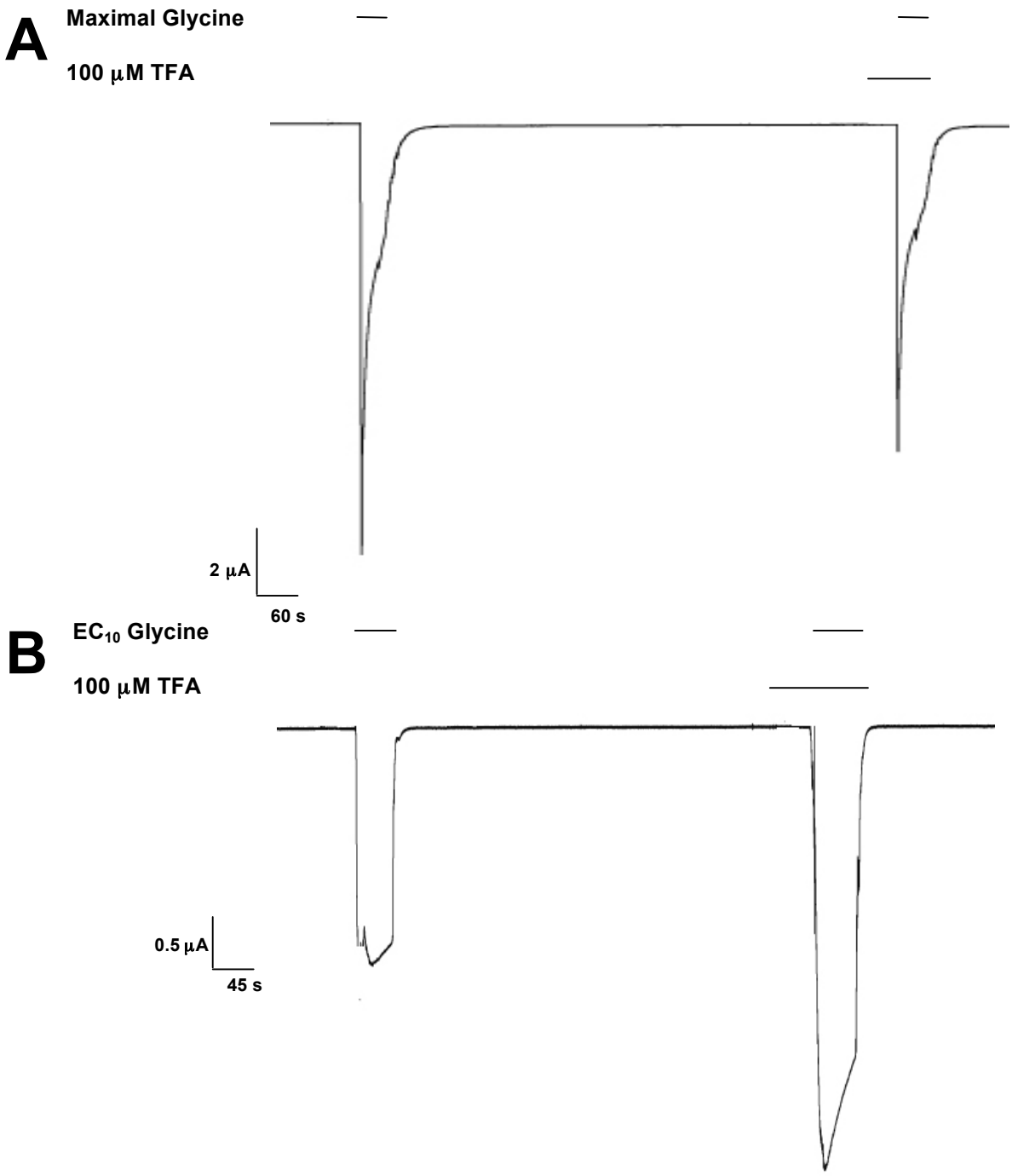

Figure 5.2 – TFA allosterically modulates the α1β GlyR

Sample tracings of the effect of 100 μM TFA on the response to maximal glycine (10 mM, *A*) and EC_{10} glycine *(B)*. For both tracings, TFA was applied alone for 30 s followed by co-application with glycine. TFA did not alter the holding current when applied alone. TFA significantly enhanced the response to EC_{10} glycine, but had no enhancing effect on the response elicited by maximal glycine.

5.3.2: TFA Differentially Modulates GlyR Subtypes

As several of the peptides generated with contaminating TFA show subunit-specific effects on the GlyR, we tested the effect of TFA on homomeric α1 channels, as well as heteromeric α2β, and α3β channels. Concentration response curves for each channel subtype are illustrated in **Figure 5.3A**. GlyR α2β channels were the least responsive to TFA, showing no effect below 0.01 mM TFA. Heteromeric α3-containing channels also did not respond to lower concentrations of TFA. For both subtypes, the maximal percent change in the EC_{10} response elicited by the tested TFA concentrations was approximately half that seen for α1β receptors. Interestingly, α1 homomeric channels were also less sensitive to modulation by low concentrations of TFA than α1β receptors (**Figure 5.3B**). However, above 0.1 mM, the effect of TFA on the homomeric channels was similar to that of α1β receptors.

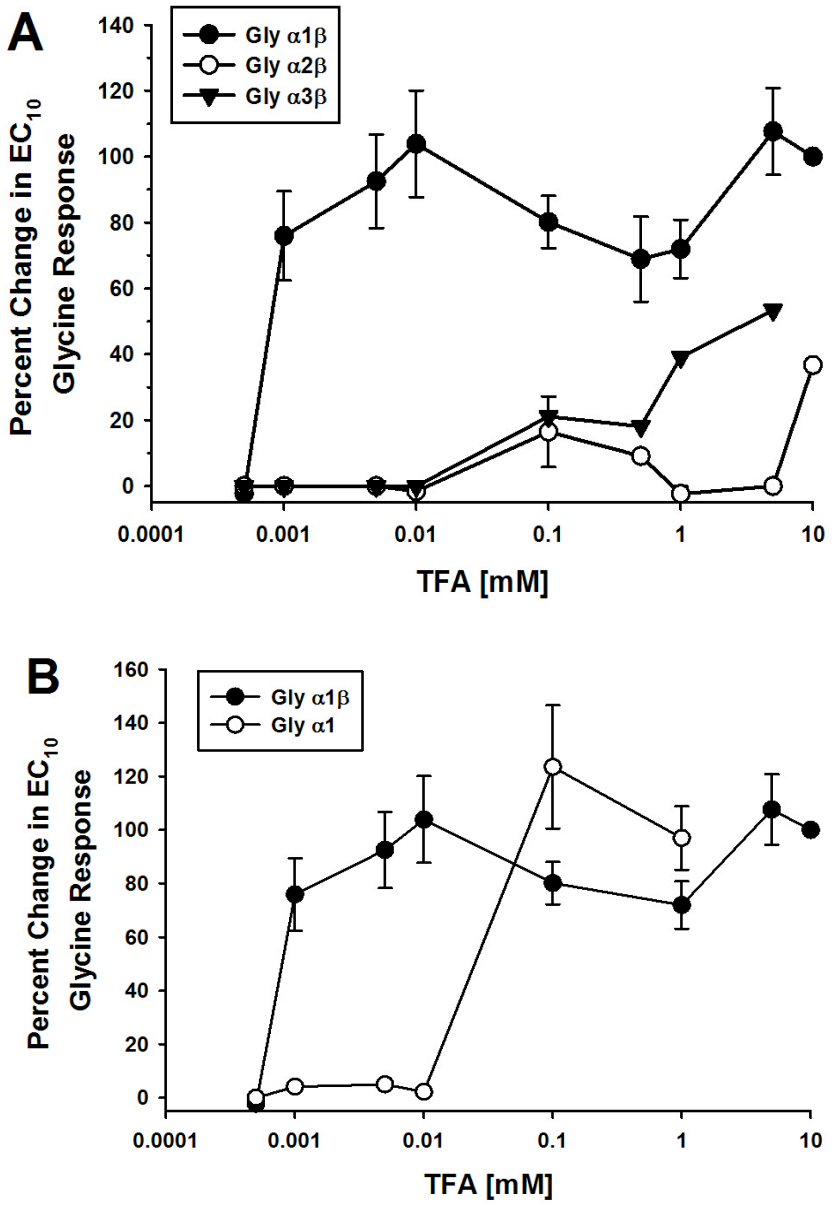

Figure 5.3 – TFA has differential effects on GlyR α subtypes

(A) Percent changes in EC_{10} glycine responses when glycine is co-applied with various concentrations of TFA (0.005 – 10 mM) on Gly α1β (black circles), α2β (white circles) and α3β (black triangles) receptors. Concentrations of TFA above 0.001 mM robustly potentiated Gly a1b channels. TFA potentiation of α2β and α3β receptors was weaker than for α1β and required a higher concentration of TFA (> 0.01 mM). *(B)* Percent change in EC_{10} glycine responses when glycine was co-applied with various concentrations of TFA (0.0005 – 10 mM) on Gly α1β (black circles) and α1 (white circles) receptors. TFA robustly potentiated both α1β heteromeric and α1 homomeric receptors; however, potentiation of α1 homomeric receptors only occurred at concentrations > 0.01 mM. Data are presented as mean ± S.E.

5.3.3: Contribution of TFA to Peptide Effects

Once a peptide synthesized as a TFA salt is exposed to a high salt solution, such as the MBS perfusion media, TFA dissociates from the peptide. Thus, any contribution of TFA to the peptide responses should be in the form of free TFA, rather than peptide-bound TFA that directly alters peptide folding or channel interaction. None of the TFA curves generated can directly account for all of the effects seen with the characterized peptides. However, this does not rule out the possibility that the peptide effects reported previously are the result of a combination of peptide and TFA actions on the GlyR, similar to the interaction noted between ethanol and zinc (McCracken et al., 2010). To conclusively delineate the role of TFA, we obtained a previously tested peptide, D12-116 synthesized as a chloride salt lacking any TFA contamination. **Figure 5.4** shows the comparison between the response to the original peptide (with unknown levels of TFA) and the new chloride salt peptides (no TFA). In the absence of TFA, the peptide still robustly (117.29 ± 13.69%) potentiated the glycine response. While the magnitude of the response differed somewhat from that of the original peptide, the difference was not significant ($p = 0.889$), suggesting that the potentiating effect is due to the peptide rather than an artifact of TFA contamination.

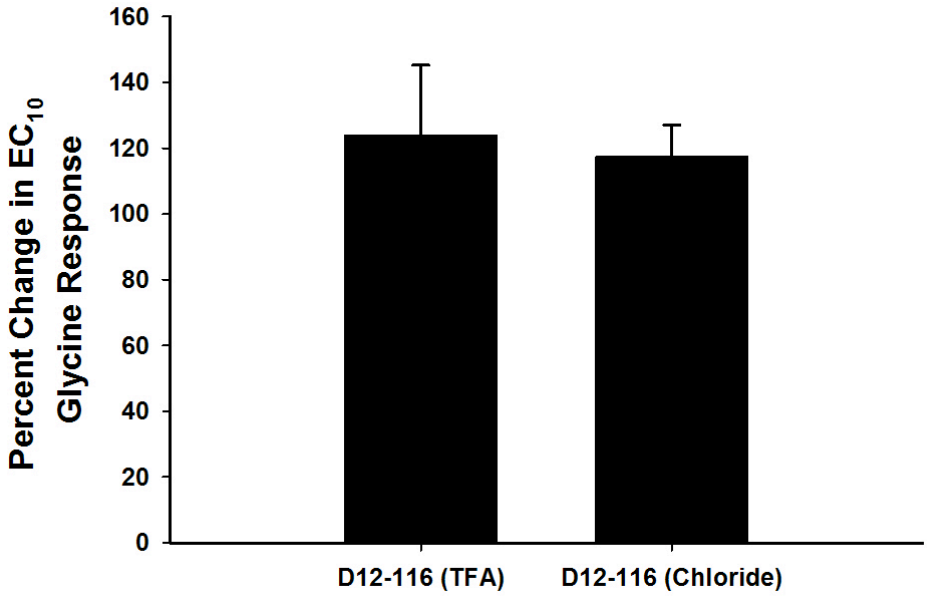

Figure 5.4 – Effects of peptide D12-116 on α1 GlyR when applied as a TFA salt or a chloride salt

Percent change in EC_{10} glycine response when glycine was co-applied with 30 μM of D12-116 purified as either a TFA salt or a chloride salt. Both forms of peptide D12-116 robustly potentiated the glycine response of Gly α1 receptors. Data are presented as mean ± S.E.

5.4 - Discussion

These experiments demonstrate that TFA, a common contaminant in synthesized peptides and a major metabolite of halothane, isofluorane and desfluorane, acts as an allosteric agent to potentiate GlyR function. While TFA, due to its highly reactive nature, is widely believed to have global, non-specific protein effects, the responses here were reversible and did not alter maximal glycine responses, indicative of a transient allosteric modulator. In addition, even high (10 mM) concentrations of TFA alone do not affect GlyR function in the absence of glycine nor did they affect any other endogenous oocyte membrane protein that contributes to the holding current. This effect, though surprising, is not unheard of. TFA has also been shown to reversibly enhance ATP-sensitive potassium (K_{ATP}) channels, an effect that is thought to be the basis for the cardioprotective effects of isofluorane (Han et al., 2001). In addition, the GlyR is a well-known target of the volatile anesthetics from which TFA is metabolized *in vivo* (Mascia et al., 1996a; Mihic et al., 1997; Zhang et al., 2003). The modulation of GlyR by TFA opens up several lines of inquiry. First, is this effect limited to the GlyR, or are other anesthetic targets also modulated by this compound? Other Cys-loop family channels in particular should be examined for their sensitivities to TFA. Secondly, is the binding site for TFA distinct from the binding site of the volatile anesthetics from which it is metabolized? A large body of research exists regarding the location of the binding site for these anesthetic compounds (Mihic et al., 1997; Roberts et al., 2006). It would be of interest to test channels with mutations at these binding sites for their responses to TFA.

Recently, TFA has received much attention as the primary breakdown product of hydrochlorofluorocarbons (HCFC) and hydrofluorocarbons (HFC), compounds that were developed as alternatives to the ozone-depleting chlorofluorocarbons (Franklin, 1993). Because HFC and HCFC compounds return to earth rather than lingering in the atmosphere, their breakdown products could have significant effects on several environmental systems (Likens et al., 1997). TFA has been of special interest due to several reports that it may be toxic to different kinds of algae and bacteria, leading to concern regarding the long-term implications of TFA accumulation (Likens et al., 1997). Thus the effect of TFA may quickly become an important area of study as the environmental levels increase.

TFA exhibits unusual differences among the various subtypes of GlyR channels. Of particular interest to the peptide work discussed in the previous chapters, TFA modulates both $\alpha 1$ and $\alpha 1\beta$ receptors, but with differing sensitivities. Both receptor types were potentiated by TFA, but $\alpha 1$ homomeric receptors require much higher TFA concentrations before the effect is seen. This may explain why the peptides tested against homomeric channels exhibited normal concentration-response curves even in the presence of contaminating TFA. For peptides tested against $\alpha 1$ receptors, contaminating concentrations of TFA above 0.01 mM would be required to see any significant effect. However, for peptides tested against the more sensitive $\alpha 1\beta$ receptors, much lower concentrations of TFA could conceivably alter channel function or the channel:peptide interaction. With that in mind, it is important to note that TFA contamination alone cannot account for all of the peptide responses at $\alpha 1\beta$ channels. For example, none of

the tested concentrations of TFA inhibited GlyR responses, while several of the peptides did. While this does not conclusively rule out the possibility of a combination of TFA and peptide effects on these channels, it does strongly suggest that at least part of the modulatory effects seen were due to the peptides themselves.

With regard to future experiments using peptide modulators, the best approach is to synthesize the peptides without the contaminating TFA. Several ion-replacement methods have been shown to successfully remove TFA, leaving the peptide as either and acetate or a chloride salt (Roux et al., 2008). This approach would circumvent the concerns that TFA contamination presents and give a more accurate prediction of the peptide effects *in vivo*.

6.0 | CONCLUSIONS AND DISCUSSION

6.1 – Overview

The CNS is a complex network of interconnected systems, each contributing to a multitude of behaviors and disease states. One method for identifying the contributions of individual systems to a given phenotype is the use of highly specific modulators. The research in this dissertation presents a novel approach for using phage display to identify peptide modulators of the GlyR, a CNS target with thus far limited options with regards to its modulation. The overall conclusion from these studies is that phage display can be used to identify peptides that bind to the GlyR and modulate channel function. This approach can be modified to identify peptides that bind with highly specificity by enhancing the stringency of the phage selection process. In addition, our studies indicate that TFA, a common contaminant in peptide synthesis and a metabolite of several volatile anesthetics, also modulates these channels, emphasizing the need to remove contaminating molecules from tested peptides.

6.2 – Identification of Peptide Modulators of the GlyR

Compared to the other members of the Cys-loop receptor family, the GlyR has a very limited pharmacological profile. With the exception of strychnine, every known GlyR modulator also interacts with other neuronal targets. Even the primary ligand, glycine, is involved in the activation of other channel types (Lynch, 2004). The lack of

specific modulators has slowed investigations into the role of GlyR in disease states and various drug-induced behaviors. In an attempt to address the lack of GlyR modulators, I modified traditional phage display techniques in order to identify peptides that bind to and modulate the GlyR. In this dissertation, several promising lead peptides were identified and a technique for isolating peptides that differentiate between closely related channel targets was developed.

The isolation of phage-expressed peptides that bind to transmembrane targets, such as ion channels, is complicated by the requirement of a membrane for functional folding. The addition of a cellular membrane contaminates the selection process with non-specific surface proteins, resulting in increased background binding and the isolation of peptides that do not show affinity for the desired target. Methods for overcoming this obstacle have been developed, including specific elution using a known ligand (Meulemans et al., 1994) and biased phage libraries based on a known ligand for the desired target (Hennecke et al., 1997). However, these approaches limit the area of the expressed target that can be investigated. Subtractive approaches have been used in which the phage are presented with cells expressing the target of interest mixed with cells without the target, followed by a cell sorting step to isolate the phage bound to the target-expressing cells (Siegel et al., 1997). I chose to approach this problem by separating the target-expressing and target free cells into two selection steps. This eliminates the need for a cell sorting step and ensures that the same background surface is presented in every round, increasing the opportunity for non-specific phage to be selected out.

Whole-cell panning makes it possible to select for ligands that bind to previously uncharacterized binding sites and cell surface structures (Lekkerkerker and Logtenberg, 1999). Given the high number of isolated peptides and the wide range of modulatory effects seen in our D12 panning **(Figure 3.1)**, it is highly likely that some of the identified peptides bind to novel modulation sights on the GlyR. For example, D12-116 does not compete with glycine at the binding site **(Figure 3.4)**, and when co-applied with ethanol, the modulatory effects were additive, suggesting that this peptide also does not bind in or over the alcohol binding site. Thus, the information obtained from these peptides can also be used to locate new biding sites for which organic modulators could conceivably be designed.

Although peptides with low nanomolar affinity have been identified directly from large libraries, the typical affinity of a linear peptide from a combinatorial library is in the high nanomolar to low micromolar range, similar to the peptides identified here (Hartley, 2002). Typically, the original selection is used to identify consensus sequences shared by several of the isolated sequences. A second library is generated using this consensus sequence and randomized flanking sequences (Szardenings et al., 1997). The sequences identified in our phage pannings were highly diverse, and no clear consensus sequence was identified (**Tables 1 and 2**). However, the alanine scan of D12-116 highlighted residues that are important for its modulatory effects (**Figure 3.5).** It would be interesting to build a phage library based on the sequence of D12-116 in which positions 6 and 12 were randomized (Weiss et al., 2000). This library could be used to identify

modified versions of D12-116 with a higher affinity for the GlyR than the original peptide.

6.3 - Peptides

While phage display has been successfully used for many years in antibody and cancer research, the field of neurobiology has been slow to embrace this technique. In part, this is due to reservations regarding the end product: peptides. As experimental agents, peptides are relatively cheap to manufacture, and can be stored for long periods of time in their lyophilized form. Therapeutically, peptides are less ideal. As a general rule, peptides do not cross the blood-brain barrier (BBB), making them difficult to administer for CNS-related purposes. However, because they represent the smallest functional part of a protein, peptides have better efficacy, selectivity, and specificity than small organic molecules (Hummel et al., 2006). In addition, peptides break down into amino acids and, due to their short half-life, do not accumulate in tissues, resulting in negligible toxicity (Loffet, 2002). Thus, if proper delivery techniques were developed, peptides would make excellent CNS modulators. To this end, several promising CNS delivery methods are being investigated. Endogenous BBB transporter systems can be utilized to carry drugs into the brain (Tamai and Tsuji, 2000). In addition, certain peptide sequences, referred to as cell penetrating peptides (CPP), have also been shown to cross membrane barriers with no additional assistance, raising the possibility that some careful modifications could carry almost any peptide across the BBB without the need for a secondary carrier (Temsamani and Vidal, 2004). Recently this technology has been used to target bioactive

peptides to brain tissue (Morris et al., 2008). For example, the TAT peptide, an 11 amino acid peptide isolated from the coat of the HIV virus, has been used to carry biologically active proteins across the BBB (Cai et al., 2011; Rapoport and Lorberboum-Galski, 2009). Nanoparticles have also been highly successful in delivering systemically administered peptides into brain tissue at pharmacologically relevant concentrations (Karatas et al., 2009). Efforts to improve the oral delivery of peptides in order to increase their marketability as therapeutic agents are also underway (Hamman et al., 2005).

6.4 – The Utility of GlyR Modulation

In addition to the experimental uses for GlyR modulators, several potential therapeutic applications exist. In the spinal chord, GlyRs mediate reciprocal inhibition in stretch reflex circuits via interneurons and the recurrent inhibition of motoneurons via Renshaw Cells. Thus, a reduction in GlyR-mediated conductance leads to changes in muscle tone (Floeter and Hallett, 1993), most notably hyperekplexia and spasticity. Spinal a3 GlyRs play an important role in inflammatory pain responses (Harvey et al., 2004), and molecules that can enhance the activity of these GlyRs would be promising candidates as anti-inflammatory analgesic compounds. Changes in the expression levels of hippocampal GlyRs have been reported in patients with temporal lobe epilepsy (Eichler et al., 2008) and in sensory processing systems, GlyRs play a role in the modulation of circuits in the central auditory pathway and the receptive fields of the retina (Lynch, 2004). Thus, a wide range of possible therapeutic uses exist for GlyR-specific modulators.

6.5 – Future Directions

The peptides identified in this dissertation have the potential to serve as excellent leads for identifying high affinity GlyR modulators. As discussed above, the development of libraries based on the peptides identified here and mutagenesis studies comparing receptor subtypes may lead to improved peptide modulation and the identification of channel residues that are critical for these effects. However, the ultimate utility in the peptides identified here lies in evaluating their effects on GlyR-related behaviors. For example, intracranial delivery of D12-116 into the NAcc would be expected to decrease ethanol consumption, based on data from the Söderpalm group (Molander et al., 2005; Molander et al., 2007).

An important aspect of the selection process developed to identify GlyR-binding peptides is how easily it can be adapted for other targets. In our lab, this panning technique has been used to identify peptides that show affinity for $GABA_A$ receptors. In addition, other labs have adapted the selection protocol to identify peptides that act as antagonists at the SK channel, and we are currently collaborating to expand this work to target the BK channel, another well-known target of alcohol actions (Treistman and Martin, 2009).

Future applications of this method may be improved by the utilization of phagemid libraries. Phagemid libraries have the advantage of being monovalent display systems, that is, only one copy of pIII carries the fused peptide. When using naïve libraries, polyvalent display and a high concentration of target will increase the chances of isolating binding peptides. Given that this was the first time phage display had been

applied using these selection conditions, I chose to start with a more conservative selection process. While the choice of a polyvalent system did yield a diverse pool of peptides with the ability to modulate the GlyR, most had a low potency. This could reflect a bias toward low affinity binding in our assay. It could also be an effect of avidity. Avidity refers to the vast increase in overall affinity that results from the summation of two or more monovalent interactions that are individually weak. The five peptide copies that exist on each phage are in close proximity, a very different situation from the application of low concentrations of free peptide. The use of a monovalent display system will bias selection toward high affinity binders and may result in the isolation of peptides with a much higher potency at the GlyR. This approach may also help resolve the lack of specificity seen in the more stringent selection for subunit specific GlyR modulators.

In summery, the research presented in this thesis describes a high throughput method for identifying novel allosteric modulators of channel function. The application of this technique to the GlyR resulted in several highly promising lead peptides, opening new avenues for the discovery of GlyR-specific modulators and the opportunity to identify novel modulatory sites on the GlyR. The ease with which this method can be adapted to other channel systems highlights the utility of phage display selection in the field of neurobiology.

Bibliography

Aguayo, L. G. and F. C. Pancetti (1994). "Ethanol modulation of the gamma-aminobutyric acidA- and glycine-activated Cl- current in cultured mouse neurons." J Pharmacol Exp Ther **270**(1): 61-69.

Ahmadi, S., S. Lippross, W. L. Neuhuber and H. U. Zeilhofer (2002). "PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons." Nat Neurosci **5**(1): 34-40.

Antognini, J. F. and K. Schwartz (1993). "Exaggerated anesthetic requirements in the preferentially anesthetized brain." Anesthesiology **79**(6): 1244-1249.

Aroeira, R. I., J. A. Ribeiro, A. M. Sebastiao and C. A. Valente (2011). "Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult." J Neurochem.

Asano, S., A. Higashitani and K. Horiuchi (1999). "Filamentous phage replication initiator protein gpII forms a covalent complex with the 5' end of the nick it introduced." Nucleic Acids Res **27**(8): 1882-1889.

Atack, J. R. (2010). "GABAA receptor alpha2/alpha3 subtype-selective modulators as potential nonsedating anxiolytics." Curr Top Behav Neurosci **2**: 331-360.

Auld, D. S. (2001). "Zinc coordination sphere in biochemical zinc sites." Biometals **14**(3-4): 271-313.

Balass, M., E. Katchalski-Katzir and S. Fuchs (1997). "The alpha-bungarotoxin binding site on the nicotinic acetylcholine receptor: analysis using a phage-epitope library." Proc Natl Acad Sci U S A **94**(12): 6054-6058.

Ballinger, M. D., V. Shyamala, L. D. Forrest, M. Deuter-Reinhard, L. V. Doyle, J. X. Wang, L. Panganiban-Lustan, J. R. Stratton, G. Apell, J. A. Winter, M. V. Doyle, S. Rosenberg and W. M. Kavanaugh (1999). "Semirational design of a potent, artificial agonist of fibroblast growth factor receptors." Nat Biotechnol **17**(12): 1199-1204.

Beato, M., P. J. Groot-Kormelink, D. Colquhoun and L. G. Sivilotti (2004). "The activation mechanism of alpha1 homomeric glycine receptors." J Neurosci **24**(4): 895-906.

Becker, C. M., W. Hoch and H. Betz (1988). "Glycine receptor heterogeneity in rat spinal cord during postnatal development." Embo J **7**(12): 3717-3726.

Beckstead, M. J., J. L. Weiner, E. I. Eger, 2nd, D. H. Gong and S. J. Mihic (2000). "Glycine and gamma-aminobutyric acid(A) receptor function is enhanced by inhaled drugs of abuse." Mol Pharmacol **57**(6): 1199-1205.

Bennett, N. J. and J. Rakonjac (2006). "Unlocking of the filamentous bacteriophage virion during infection is mediated by the C domain of pIII." J Mol Biol **356**(2): 266-273.

Berg, T. (2003). "Modulation of protein-protein interactions with small organic molecules." Angew Chem Int Ed Engl **42**(22): 2462-2481.

Bertaccini, E. J., J. Shapiro, D. L. Brutlag and J. R. Trudell (2005). "Homology modeling of a human glycine alpha 1 receptor reveals a plausible anesthetic binding site." J Chem Inf Model **45**(1): 128-135.

Birinyi, A., D. Parker, M. Antal and O. Shupliakov (2001). "Zinc co-localizes with GABA and glycine in synapses in the lamprey spinal cord." J Comp Neurol **433**(2): 208-221.

Bloomenthal, A. B., E. Goldwater, D. B. Pritchett and N. L. Harrison (1994). "Biphasic modulation of the strychnine-sensitive glycine receptor by Zn2+." Mol Pharmacol **46**(6): 1156-1159.

Bocquet, N., H. Nury, M. Baaden, C. Le Poupon, J. P. Changeux, M. Delarue and P. J. Corringer (2009). "X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation." Nature **457**(7225): 111-114.

Boeke, J. D., P. Model and N. D. Zinder (1982). "Effects of bacteriophage f1 gene III protein on the host cell membrane." Mol Gen Genet **186**(2): 185-192.

Bormann, J., N. Rundstrom, H. Betz and D. Langosch (1993). "Residues within transmembrane segment M2 determine chloride conductance of glycine receptor homo- and hetero-oligomers." Embo J **12**(10): 3729-3737.

Bradbury, A. R. and J. D. Marks (2004). "Antibodies from phage antibody libraries." J Immunol Methods **290**(1-2): 29-49.

Brams, M., A. Pandya, D. Kuzmin, R. van Elk, L. Krijnen, J. L. Yakel, V. Tsetlin, A. B. Smit and C. Ulens (2011). "A structural and mutagenic blueprint for molecular

recognition of strychnine and d-tubocurarine by different cys-loop receptors." PLoS Biol **9**(3): e1001034.

Brejc, K., W. J. van Dijk, R. V. Klaassen, M. Schuurmans, J. van Der Oost, A. B. Smit and T. K. Sixma (2001). "Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors." Nature **411**(6835): 269-276.

Buck, K. J., A. M. Allan and R. A. Harris (1989). "Fluidization of brain membranes by A2C does not produce anesthesia and does not augment muscimol-stimulated 36Cl-influx." Eur J Pharmacol **160**(3): 359-367.

Cabibbo, A., E. Sporeno, C. Toniatti, S. Altamura, R. Savino, G. Paonessa and G. Ciliberto (1995). "Monovalent phage display of human interleukin (hIL)-6: selection of superbinder variants from a complex molecular repertoire in the hIL-6 D-helix." Gene **167**(1-2): 41-47.

Cai, B., Y. Lin, X. H. Xue, L. Fang, N. Wang and Z. Y. Wu (2011). "TAT-mediated delivery of neuroglobin protects against focal cerebral ischemia in mice." Exp Neurol **227**(1): 224-231.

Cain, S. A., C. F. Ratcliffe, D. M. Williams, V. Harris and P. N. Monk (2000). "Analysis of receptor/ligand interactions using whole-molecule randomly-mutated ligand libraries." J Immunol Methods **245**(1-2): 139-145.

Celentano, J. J., T. T. Gibbs and D. H. Farb (1988). "Ethanol potentiates GABA- and glycine-induced chloride currents in chick spinal cord neurons." Brain Res **455**(2): 377-380.

Chen, X., T. I. Webb and J. W. Lynch (2009). "The M4 transmembrane segment contributes to agonist efficacy differences between alpha1 and alpha3 glycine receptors." Mol Membr Biol **26**(5): 321-332.

Cheng, M. H., R. D. Coalson and M. Cascio (2008). "Molecular dynamics simulations of ethanol binding to the transmembrane domain of the glycine receptor: implications for the channel potentiation mechanism." Proteins **71**(2): 972-981.

Cheng, S. C. and E. A. Brunner (1985). "Inducing anesthesia with a GABA analog, THIP." Anesthesiology **63**(2): 147-151.

Chi, E. Y., S. Krishnan, T. W. Randolph and J. F. Carpenter (2003). "Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation." Pharm Res **20**(9): 1325-1336.

Clackson, T., H. R. Hoogenboom, A. D. Griffiths and G. Winter (1991). "Making antibody fragments using phage display libraries." Nature **352**(6336): 624-628.

Clackson, T. and J. A. Wells (1994). "In vitro selection from protein and peptide libraries." Trends Biotechnol **12**(5): 173-184.

Clarke, M., L. Maddera, R. L. Harris and P. M. Silverman (2008). "F-pili dynamics by live-cell imaging." Proc Natl Acad Sci U S A **105**(46): 17978-17981.

Click, E. M. and R. E. Webster (1997). "Filamentous phage infection: required interactions with the TolA protein." J Bacteriol **179**(20): 6464-6471.

Cohen, E. N. (1971). "Metabolism of the volatile anesthetics." Anesthesiology **35**(2): 193-202.

Colquhoun, D. (1998). "Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors." Br J Pharmacol **125**(5): 924-947.

Cornish, J., K. E. Callon, C. Q. Lin, C. L. Xiao, T. B. Mulvey, G. J. Cooper and I. R. Reid (1999). "Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes." Am J Physiol **277**(5 Pt 1): E779-783.

Crabbe, J. C., T. J. Phillips, R. A. Harris, M. A. Arends and G. F. Koob (2006). "Alcohol-related genes: contributions from studies with genetically engineered mice." Addict Biol **11**(3-4): 195-269.

Crawford, D. K., D. I. Perkins, J. R. Trudell, E. J. Bertaccini, D. L. Davies and R. L. Alkana (2008). "Roles for loop 2 residues of alpha1 glycine receptors in agonist activation." J Biol Chem **283**(41): 27698-27706.

Crawford, D. K., J. R. Trudell, E. J. Bertaccini, K. Li, D. L. Davies and R. L. Alkana (2007). "Evidence that ethanol acts on a target in Loop 2 of the extracellular domain of alpha1 glycine receptors." J Neurochem **102**(6): 2097-2109.

Cunningham, B. C. and J. A. Wells (1989). "High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis." Science **244**(4908): 1081-1085.

Cwirla, S. E., P. Balasubramanian, D. J. Duffin, C. R. Wagstrom, C. M. Gates, S. C. Singer, A. M. Davis, R. L. Tansik, L. C. Mattheakis, C. M. Boytos, P. J. Schatz, D. P. Baccanari, N. C. Wrighton, R. W. Barrett and W. J. Dower (1997). "Peptide agonist of the

thrombopoietin receptor as potent as the natural cytokine." Science **276**(5319): 1696-1699.

Cwirla, S. E., E. A. Peters, R. W. Barrett and W. J. Dower (1990). "Peptides on phage: a vast library of peptides for identifying ligands." Proc Natl Acad Sci U S A **87**(16): 6378-6382.

Daly, E. C. and M. H. Aprison (1974). "Distribution of serine hydroxymethyltransferase and glycine transaminase in several areas of the central nervous system of the rat." J Neurochem **22**(6): 877-885.

Davidoff, R. A., R. P. Shank, L. T. Graham, Jr., M. H. Aprison and R. Werman (1967). "Association of glycine with spinal interneurones." Nature **214**(5089): 680-681.

Davies, P. A., W. Wang, T. G. Hales and E. F. Kirkness (2003). "A novel class of ligand-gated ion channel is activated by Zn2+." J Biol Chem **278**(2): 712-717.

Delaney, A. J., A. Esmaeili, P. L. Sedlak, J. W. Lynch and P. Sah (2010). "Differential expression of glycine receptor subunits in the rat basolateral and central amygdala." Neurosci Lett **469**(2): 237-242.

Devlin, J. J., L. C. Panganiban and P. E. Devlin (1990). "Random peptide libraries: a source of specific protein binding molecules." Science **249**(4967): 404-406.

Di Chiara, G. and A. Imperato (1988). "Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats." Proc Natl Acad Sci U S A **85**(14): 5274-5278.

Doorbar, J. and G. Winter (1994). "Isolation of a peptide antagonist to the thrombin receptor using phage display." J Mol Biol **244**(4): 361-369.

Duan, L., J. Yang and M. M. Slaughter (2009). "Caffeine inhibition of ionotropic glycine receptors." J Physiol **587**(Pt 16): 4063-4075.

Eggers, E. D., J. A. O'Brien and A. J. Berger (2000). "Developmental changes in the modulation of synaptic glycine receptors by ethanol." J Neurophysiol **84**(5): 2409-2416.

Eichler, S. A., S. Kirischuk, R. Juttner, P. K. Schafermeier, P. Legendre, T. N. Lehmann, T. Gloveli, R. Grantyn and J. C. Meier (2008). "Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy." J Cell Mol Med **12**(6B): 2848-2866.

Endemann, H. and P. Model (1995). "Location of filamentous phage minor coat proteins in phage and in infected cells." J Mol Biol **250**(4): 496-506.

Engblom, A. C. and K. E. Akerman (1991). "Effect of ethanol on gamma-aminobutyric acid and glycine receptor-coupled Cl- fluxes in rat brain synaptoneurosomes." J Neurochem **57**(2): 384-390.

Ericson, M., R. B. Clarke, P. Chau, L. Adermark and B. Soderpalm (2010). "beta-Alanine elevates dopamine levels in the rat nucleus accumbens: antagonism by strychnine." Amino Acids **38**(4): 1051-1055.

Ericson, M., A. Molander, E. Lof, J. A. Engel and B. Soderpalm (2003). "Ethanol elevates accumbal dopamine levels via indirect activation of ventral tegmental nicotinic acetylcholine receptors." Eur J Pharmacol **467**(1-3): 85-93.

Ericson, M., A. Molander, R. Stomberg and B. Soderpalm (2006). "Taurine elevates dopamine levels in the rat nucleus accumbens; antagonism by strychnine." Eur J Neurosci **23**(12): 3225-3229.

Findlay, G. S., R. Phelan, M. T. Roberts, G. E. Homanics, S. E. Bergeson, G. F. Lopreato, S. J. Mihic, Y. A. Blednov and R. A. Harris (2003). "Glycine receptor knock-in mice and hyperekplexia-like phenotypes: comparisons with the null mutant." J Neurosci **23**(22): 8051-8059.

Findlay, G. S., M. J. Wick, M. P. Mascia, D. Wallace, G. W. Miller, R. A. Harris and Y. A. Blednov (2002). "Transgenic expression of a mutant glycine receptor decreases alcohol sensitivity of mice." J Pharmacol Exp Ther **300**(2): 526-534.

Floeter, M. K. and M. Hallett (1993). "Glycine receptors: a startling connection." Nat Genet **5**(4): 319-320.

Franks, N. P. (2006). "Molecular targets underlying general anaesthesia." Br J Pharmacol **147 Suppl 1**: S72-81.

Franks, N. P. and W. R. Lieb (1985). "Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects." Nature **316**(6026): 349-351.

Franks, N. P. and W. R. Lieb (1997a). "Inhibitory synapses. Anaesthetics set their sites on ion channels." Nature **389**(6649): 334-335.

Franks, N. P. and W. R. Lieb (1997b). "Selectivity of general anesthetics: a new dimension." Nat Med **3**(4): 377-378.

Frederickson, C. J., L. J. Giblin, A. Krezel, D. J. McAdoo, R. N. Mueller, Y. Zeng, R. V. Balaji, R. Masalha, R. B. Thompson, C. A. Fierke, J. M. Sarvey, M. de Valdenebro, D. S. Prough and M. H. Zornow (2006). "Concentrations of extracellular free zinc (pZn)e in the central nervous system during simple anesthetization, ischemia and reperfusion." Exp Neurol **198**(2): 285-293.

Freshney, R. I. (2002). "Cell line provenance." Cytotechnology **39**(2): 55-67.

Frischknecht, A. L. and L. J. Frink (2006). "Alcohols reduce lateral membrane pressures: predictions from molecular theory." Biophys J **91**(11): 4081-4090.

Fritschy, J. M., R. J. Harvey and G. Schwarz (2008). "Gephyrin: where do we stand, where do we go?" Trends Neurosci **31**(5): 257-264.

Fuentealba, J., B. Munoz, G. Yevenes, G. Moraga-Cid, C. Perez, L. Guzman, J. M. Rigo and L. G. Aguayo (2011). "Potentiation and inhibition of glycine receptors by tutin." Neuropharmacology **60**(2-3): 453-459.

Gage, P. W. and B. Robertson (1985). "Prolongation of inhibitory postsynaptic currents by pentobarbitone, halothane and ketamine in CA1 pyramidal cells in rat hippocampus." Br J Pharmacol **85**(3): 675-681.

Garcia, M. C. (2005). "The effect of the mobile phase additives on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography-electrospray mass spectrometry." J Chromatogr B Analyt Technol Biomed Life Sci **825**(2): 111-123.

Gatto, G. J., W. J. McBride, J. M. Murphy, L. Lumeng and T. K. Li (1994). "Ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats." Alcohol **11**(6): 557-564.

Gerding, M. A., Y. Ogata, N. D. Pecora, H. Niki and P. A. de Boer (2007). "The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli." Mol Microbiol **63**(4): 1008-1025.

Gilbert, D. F., R. Islam, T. Lynagh, J. W. Lynch and T. I. Webb (2009). "High Throughput Techniques for Discovering New Glycine Receptor Modulators and their Binding Sites." Front Mol Neurosci **2**: 17.

Gill, J. K., M. Savolainen, G. T. Young, R. Zwart, E. Sher and N. S. Millar (2011). "Agonist activation of {alpha}7 nicotinic acetylcholine receptors via an allosteric transmembrane site." Proc Natl Acad Sci U S A **108**(14): 5867-5872.

Glaser-Wuttke, G., J. Keppner and I. Rasched (1989). "Pore-forming properties of the adsorption protein of filamentous phage fd." Biochim Biophys Acta **985**(3): 239-247.

Goldstein, D. B. and J. H. Chin (1981). "Interaction of ethanol with biological membranes." Fed Proc **40**(7): 2073-2076.

Goodson, R. J., M. V. Doyle, S. E. Kaufman and S. Rosenberg (1994). "High-affinity urokinase receptor antagonists identified with bacteriophage peptide display." Proc Natl Acad Sci U S A **91**(15): 7129-7133.

Gray, C. W., R. S. Brown and D. A. Marvin (1981). "Adsorption complex of filamentous fd virus." J Mol Biol **146**(4): 621-627.

Grenningloh, G., E. Gundelfinger, B. Schmitt, H. Betz, M. G. Darlison, E. A. Barnard, P. R. Schofield and P. H. Seeburg (1987a). "Glycine vs GABA receptors." Nature **330**(6143): 25-26.

Grenningloh, G., A. Rienitz, B. Schmitt, C. Methfessel, M. Zensen, K. Beyreuther, E. D. Gundelfinger and H. Betz (1987b). "The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors." Nature **328**(6127): 215-220.

Grosman, C., M. Zhou and A. Auerbach (2000). "Mapping the conformational wave of acetylcholine receptor channel gating." Nature **403**(6771): 773-776.

Grudzinska, J., R. Schemm, S. Haeger, A. Nicke, G. Schmalzing, H. Betz and B. Laube (2005). "The beta subunit determines the ligand binding properties of synaptic glycine receptors." Neuron **45**(5): 727-739.

Grunert, U. (2000). "Distribution of GABA and glycine receptors on bipolar and ganglion cells in the mammalian retina." Microsc Res Tech **50**(2): 130-140.

Gut, J., U. Christen, N. Frey, V. Koch and D. Stoffler (1995). "Molecular mimicry in halothane hepatitis: biochemical and structural characterization of lipoylated autoantigens." Toxicology **97**(1-3): 199-224.

Guzman, L., G. Moraga-Cid, A. Avila, M. Figueroa, G. E. Yevenes, J. Fuentealba and L. G. Aguayo (2009). "Blockade of ethanol-induced potentiation of glycine receptors by a

peptide that interferes with Gbetagamma binding." J Pharmacol Exp Ther **331**(3): 933-939.

Haigh, N. G. and R. E. Webster (1998). "The major coat protein of filamentous bacteriophage f1 specifically pairs in the bacterial cytoplasmic membrane." J Mol Biol **279**(1): 19-29.

Hamman, J. H., G. M. Enslin and A. F. Kotze (2005). "Oral delivery of peptide drugs: barriers and developments." BioDrugs **19**(3): 165-177.

Han, J., N. Kim and E. Kim (2001). "Trifluoroacetic acid activates ATP-sensitive K(+) channels in rabbit ventricular myocytes." Biochem Biophys Res Commun **285**(5): 1136-1142.

Harris, R. A., S. J. Mihic, J. E. Dildy-Mayfield and T. K. Machu (1995). "Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition." Faseb J **9**(14): 1454-1462.

Hartley, O. (2002). "The use of phage display in the study of receptors and their ligands." J Recept Signal Transduct Res **22**(1-4): 373-392.

Harvey, R. J., U. B. Depner, H. Wassle, S. Ahmadi, C. Heindl, H. Reinold, T. G. Smart, K. Harvey, B. Schutz, O. M. Abo-Salem, A. Zimmer, P. Poisbeau, H. Welzl, D. P. Wolfer, H. Betz, H. U. Zeilhofer and U. Muller (2004). "GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization." Science **304**(5672): 884-887.

Harvey, R. J., P. Thomas, C. H. James, A. Wilderspin and T. G. Smart (1999). "Identification of an inhibitory Zn2+ binding site on the human glycine receptor alpha1 subunit." J Physiol **520 Pt 1**: 53-64.

Hawthorne, R. and J. W. Lynch (2005). "A picrotoxin-specific conformational change in the glycine receptor M2-M3 loop." J Biol Chem **280**(43): 35836-35843.

Heidmann, T., A. Sobel, J. L. Popot and J. P. Changeux (1980). "Reconstitution of a functional acetylcholine receptor. Conservation of the conformational and allosteric transitions and recovery of the permeability response; role of lipids." Eur J Biochem **110**(1): 35-55.

Hennecke, M., A. Kola, M. Baensch, A. Wrede, A. Klos, W. Bautsch and J. Kohl (1997). "A selection system to study C5a-C5a-receptor interactions: phage display of a novel C5a anaphylatoxin, Fos-C5aAla27." Gene **184**(2): 263-272.

Hibbs, R. E. and E. Gouaux (2011). "Principles of activation and permeation in an anion-selective Cys-loop receptor." Nature.

Hilf, R. J. and R. Dutzler (2008). "X-ray structure of a prokaryotic pentameric ligand-gated ion channel." Nature **452**(7185): 375-379.

Hirata, H., E. Carta, I. Yamanaka, R. J. Harvey and J. Y. Kuwada (2009). "Defective glycinergic synaptic transmission in zebrafish motility mutants." Front Mol Neurosci **2**: 26.

Hirzel, K., U. Muller, A. T. Latal, S. Hulsmann, J. Grudzinska, M. W. Seeliger, H. Betz and B. Laube (2006). "Hyperekplexia phenotype of glycine receptor alpha1 subunit mutant mice identifies Zn(2+) as an essential endogenous modulator of glycinergic neurotransmission." Neuron **52**(4): 679-690.

Hitt, B. A., R. I. Mazze, M. J. Cousins, H. N. Edmunds, G. A. Barr and J. R. Trudell (1974). "Metabolism of isoflurane in Fischer 344 rats and man." Anesthesiology **40**(1): 62-67.

Holliger, P. and L. Riechmann (1997). "A conserved infection pathway for filamentous bacteriophages is suggested by the structure of the membrane penetration domain of the minor coat protein g3p from phage fd." Structure **5**(2): 265-275.

Holliger, P., L. Riechmann and R. L. Williams (1999). "Crystal structure of the two N-terminal domains of g3p from filamentous phage fd at 1.9 A: evidence for conformational lability." J Mol Biol **288**(4): 649-657.

Hoogenboom, H. R. and G. Winter (1992). "By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro." J Mol Biol **227**(2): 381-388.

Horabin, J. I. and R. E. Webster (1988). "An amino acid sequence which directs membrane insertion causes loss of membrane potential." J Biol Chem **263**(23): 11575-11583.

Houamed, K. M., G. Bilbe, T. G. Smart, A. Constanti, D. A. Brown, E. A. Barnard and B. M. Richards (1984). "Expression of functional GABA, glycine and glutamate receptors in Xenopus oocytes injected with rat brain mRNA." Nature **310**(5975): 318-321.

Huidobro-Toro, J. P., V. Bleck, A. M. Allan and R. A. Harris (1987). "Neurochemical actions of anesthetic drugs on the gamma-aminobutyric acid receptor-chloride channel complex." J Pharmacol Exp Ther **242**(3): 963-969.

Hummel, G., U. Reineke and U. Reimer (2006). "Translating peptides into small molecules." Mol Biosyst **2**(10): 499-508.

Hyytia, P. and G. F. Koob (1995). "GABAA receptor antagonism in the extended amygdala decreases ethanol self-administration in rats." Eur J Pharmacol **283**(1-3): 151-159.

Ivanova, E., U. Muller and H. Wassle (2006). "Characterization of the glycinergic input to bipolar cells of the mouse retina." Eur J Neurosci **23**(2): 350-364.

Jeong, H. J., I. S. Jang, A. J. Moorhouse and N. Akaike (2003). "Activation of presynaptic glycine receptors facilitates glycine release from presynaptic terminals synapsing onto rat spinal sacral dorsal commissural nucleus neurons." J Physiol **550**(Pt 2): 373-383.

Jonsson, S., N. Kerekes, P. Hyytia, M. Ericson and B. Soderpalm (2009). "Glycine receptor expression in the forebrain of male AA/ANA rats." Brain Res **1305 Suppl**: S27-36.

Kallen, K. J., J. Grotzinger and S. Rose-John (2000). "New perspectives on the design of cytokines and growth factors." Trends Biotechnol **18**(11): 455-461.

Karatas, H., Y. Aktas, Y. Gursoy-Ozdemir, E. Bodur, M. Yemisci, S. Caban, A. Vural, O. Pinarbasli, Y. Capan, E. Fernandez-Megia, R. Novoa-Carballal, R. Riguera, K. Andrieux, P. Couvreur and T. Dalkara (2009). "A nanomedicine transports a peptide caspase-3 inhibitor across the blood-brain barrier and provides neuroprotection." J Neurosci **29**(44): 13761-13769.

Kay, B. K., N. B. Adey, Y. S. He, J. P. Manfredi, A. H. Mataragnon and D. M. Fowlkes (1993). "An M13 phage library displaying random 38-amino-acid peptides as a source of novel sequences with affinity to selected targets." Gene **128**(1): 59-65.

Kim, M. S., A. Repp and D. P. Smith (1998). "LUSH odorant-binding protein mediates chemosensory responses to alcohols in Drosophila melanogaster." Genetics **150**(2): 711-721.

Krasowski, M. D. and N. L. Harrison (2000). "The actions of ether, alcohol and alkane general anaesthetics on GABAA and glycine receptors and the effects of TM2 and TM3 mutations." Br J Pharmacol **129**(4): 731-743.

Kruse, S. W., R. Zhao, D. P. Smith and D. N. Jones (2003). "Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from Drosophila melanogaster." Nat Struct Biol **10**(9): 694-700.

Kudo, Y., H. Niwa, A. Tanaka and K. Yamada (1984). "Actions of picrotoxinin and related compounds on the frog spinal cord: the role of a hydroxyl-group at the 6-position in antagonizing the actions of amino acids and presynaptic inhibition." Br J Pharmacol **81**(2): 373-380.

Kuhse, J., B. Laube, D. Magalei and H. Betz (1993). "Assembly of the inhibitory glycine receptor: identification of amino acid sequence motifs governing subunit stoichiometry." Neuron **11**(6): 1049-1056.

Kunkel, T. A. (1985). "Rapid and efficient site-specific mutagenesis without phenotypic selection." Proc Natl Acad Sci U S A **82**(2): 488-492.

Kunkel, T. A., J. D. Roberts and R. A. Zakour (1987). "Rapid and efficient site-specific mutagenesis without phenotypic selection." Methods Enzymol **154**: 367-382.

Langosch, D., L. Thomas and H. Betz (1988). "Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer." Proc Natl Acad Sci U S A **85**(19): 7394-7398.

Laube, B., J. Kuhse and H. Betz (2000). "Kinetic and mutational analysis of Zn2+ modulation of recombinant human inhibitory glycine receptors." J Physiol **522 Pt 2**: 215-230.

Legendre, P. (2001). "The glycinergic inhibitory synapse." Cell Mol Life Sci **58**(5-6): 760-793.

Lekkerkerker, A. and T. Logtenberg (1999). "Phage antibodies against human dendritic cell subpopulations obtained by flow cytometry-based selection on freshly isolated cells." J Immunol Methods **231**(1-2): 53-63.

Leshner, A. I. and G. F. Koob (1999). "Drugs of abuse and the brain." Proc Assoc Am Physicians **111**(2): 99-108.

Lester, H. A., M. I. Dibas, D. S. Dahan, J. F. Leite and D. A. Dougherty (2004). "Cys-loop receptors: new twists and turns." Trends Neurosci **27**(6): 329-336.

Lewis, T. M., L. G. Sivilotti, D. Colquhoun, R. M. Gardiner, R. Schoepfer and M. Rees (1998). "Properties of human glycine receptors containing the hyperekplexia

mutation alpha1(K276E), expressed in Xenopus oocytes." J Physiol **507 (Pt 1)**: 25-40.

Li, M., W. Yu, C. H. Chen, S. Cwirla, E. Whitehorn, E. Tate, R. Raab, M. Bremer and B. Dower (1996). "In vitro selection of peptides acting at a new site of NMDA glutamate receptors." Nat Biotechnol **14**(8): 986-991.

Li, Z., A. Zharikova, J. Bastian, L. Esperon, N. Hebert, C. Mathes, N. E. Rowland and J. Peris (2008). "High temporal resolution of amino acid levels in rat nucleus accumbens during operant ethanol self-administration: involvement of elevated glycine in anticipation." J Neurochem **106**(1): 170-181.

Likens, G. E., S. L. Tartowski, T. W. Berger, D. G. Richey, C. T. Driscoll, H. G. Frank and A. Klein (1997). "Transport and fate of trifluoroacetate in upland forest and wetland ecosystems." Proc Natl Acad Sci U S A **94**(9): 4499-4503.

Lobo, I. A., M. P. Mascia, J. R. Trudell and R. A. Harris (2004). "Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics." J Biol Chem **279**(32): 33919-33927.

Loffet, A. (2002). "Peptides as drugs: is there a market?" J Pept Sci **8**(1): 1-7.

Lopez, J. and R. E. Webster (1985). "Assembly site of bacteriophage f1 corresponds to adhesion zones between the inner and outer membranes of the host cell." J Bacteriol **163**(3): 1270-1274.

Lovinger, D. M. (1997). "Alcohols and neurotransmitter gated ion channels: past, present and future." Naunyn Schmiedebergs Arch Pharmacol **356**(3): 267-282.

Lubkowski, J., F. Hennecke, A. Pluckthun and A. Wlodawer (1998). "The structural basis of phage display elucidated by the crystal structure of the N-terminal domains of g3p." Nat Struct Biol **5**(2): 140-147.

Lubkowski, J., F. Hennecke, A. Pluckthun and A. Wlodawer (1999). "Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA." Structure **7**(6): 711-722.

Lynch, J. W. (2004). "Molecular structure and function of the glycine receptor chloride channel." Physiol Rev **84**(4): 1051-1095.

Lynch, J. W. (2009). "Native glycine receptor subtypes and their physiological roles." Neuropharmacology **56**(1): 303-309.

Lynch, J. W., P. Jacques, K. D. Pierce and P. R. Schofield (1998). "Zinc potentiation of the glycine receptor chloride channel is mediated by allosteric pathways." J Neurochem **71**(5): 2159-2168.

Lynch, J. W., S. Rajendra, P. H. Barry and P. R. Schofield (1995). "Mutations affecting the glycine receptor agonist transduction mechanism convert the competitive antagonist, picrotoxin, into an allosteric potentiator." J Biol Chem **270**(23): 13799-13806.

Lynch, J. W., S. Rajendra, K. D. Pierce, C. A. Handford, P. H. Barry and P. R. Schofield (1997). "Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel." Embo J **16**(1): 110-120.

Ma, T. G., Y. H. Ling, G. D. McClure and M. T. Tseng (1990). "Effects of trifluoroacetic acid, a halothane metabolite, on C6 glioma cells." J Toxicol Environ Health **31**(2): 147-158.

Maksay, G. (2009). "Ligand-gated pentameric ion channels, from binding to gating." Curr Mol Pharmacol **2**(3): 253-262.

Malik, P., T. D. Terry, F. Bellintani and R. N. Perham (1998). "Factors limiting display of foreign peptides on the major coat protein of filamentous bacteriophage capsids and a potential role for leader peptidase." FEBS Lett **436**(2): 263-266.

Malosio, M. L., B. Marqueze-Pouey, J. Kuhse and H. Betz (1991). "Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain." Embo J **10**(9): 2401-2409.

Marvin, D. A. (1998). "Filamentous phage structure, infection and assembly." Curr Opin Struct Biol **8**(2): 150-158.

Marvin, D. A. and B. Hohn (1969). "Filamentous bacterial viruses." Bacteriol Rev **33**(2): 172-209.

Marvizon, J. C., J. Vazquez, M. Garcia Calvo, F. Mayor, Jr., A. Ruiz Gomez, F. Valdivieso and J. Benavides (1986). "The glycine receptor: pharmacological studies and mathematical modeling of the allosteric interaction between the glycine- and strychnine-binding sites." Mol Pharmacol **30**(6): 590-597.

Mascia, M. P., T. K. Machu and R. A. Harris (1996a). "Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics." Br J Pharmacol **119**(7): 1331-1336.

Mascia, M. P., S. J. Mihic, C. F. Valenzuela, P. R. Schofield and R. A. Harris (1996b). "A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors." Mol Pharmacol **50**(2): 402-406.

Mascia, M. P., J. R. Trudell and R. A. Harris (2000). "Specific binding sites for alcohols and anesthetics on ligand-gated ion channels." Proc Natl Acad Sci U S A **97**(16): 9305-9310.

Mascia, M. P., M. J. Wick, L. D. Martinez and R. A. Harris (1998). "Enhancement of glycine receptor function by ethanol: role of phosphorylation." Br J Pharmacol **125**(2): 263-270.

Mathie, A., G. L. Sutton, C. E. Clarke and E. L. Veale (2006). "Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability." Pharmacol Ther **111**(3): 567-583.

Matsubayashi, H., M. Alkondon, E. F. Pereira, K. L. Swanson and E. X. Albuquerque (1998). "Strychnine: a potent competitive antagonist of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal neurons." J Pharmacol Exp Ther **284**(3): 904-913.

May, L. T., V. A. Avlani, P. M. Sexton and A. Christopoulos (2004). "Allosteric modulation of G protein-coupled receptors." Curr Pharm Des **10**(17): 2003-2013.

McCafferty, J., A. D. Griffiths, G. Winter and D. J. Chiswell (1990). "Phage antibodies: filamentous phage displaying antibody variable domains." Nature **348**(6301): 552-554.

McCool, B. A. and S. K. Botting (2000). "Characterization of strychnine-sensitive glycine receptors in acutely isolated adult rat basolateral amygdala neurons." Brain Res **859**(2): 341-351.

McCracken, L. M., J. R. Trudell, B. E. Goldstein, R. A. Harris and S. J. Mihic (2010). "Zinc enhances ethanol modulation of the alpha1 glycine receptor." Neuropharmacology **58**(3): 676-681.

Meier, J. C., C. Henneberger, I. Melnick, C. Racca, R. J. Harvey, U. Heinemann, V. Schmieden and R. Grantyn (2005). "RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency." Nat Neurosci **8**(6): 736-744.

Messing, J., B. Gronenborn, B. Muller-Hill and P. Hans Hopschneider (1977). "Filamentous coliphage M13 as a cloning vehicle: insertion of a HindII fragment of

the lac regulatory region in M13 replicative form in vitro." Proc Natl Acad Sci U S A **74**(9): 3642-3646.

Meulemans, E. V., R. Slobbe, P. Wasterval, F. C. Ramaekers and G. J. van Eys (1994). "Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody." J Mol Biol **244**(4): 353-360.

Meyer, T. F. and K. Geider (1982). "Enzymatic synthesis of bacteriophage fd viral DNA." Nature **296**(5860): 828-832.

Michel, B. and N. D. Zinder (1989). "Translational repression in bacteriophage f1: characterization of the gene V protein target on the gene II mRNA." Proc Natl Acad Sci U S A **86**(11): 4002-4006.

Mihic, S. J. and R. A. Harris (1996). "Inhibition of rho1 receptor GABAergic currents by alcohols and volatile anesthetics." J Pharmacol Exp Ther **277**(1): 411-416.

Mihic, S. J., P. J. Whiting, R. L. Klein, K. A. Wafford and R. A. Harris (1994). "A single amino acid of the human gamma-aminobutyric acid type A receptor gamma 2 subunit determines benzodiazepine efficacy." J Biol Chem **269**(52): 32768-32773.

Mihic, S. J., Q. Ye, M. J. Wick, V. V. Koltchine, M. D. Krasowski, S. E. Finn, M. P. Mascia, C. F. Valenzuela, K. K. Hanson, E. P. Greenblatt, R. A. Harris and N. L. Harrison (1997). "Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors." Nature **389**(6649): 385-389.

Miller, P. S., M. Beato, R. J. Harvey and T. G. Smart (2005a). "Molecular determinants of glycine receptor alphabeta subunit sensitivities to Zn2+-mediated inhibition." J Physiol **566**(Pt 3): 657-670.

Miller, P. S., H. M. Da Silva and T. G. Smart (2005b). "Molecular basis for zinc potentiation at strychnine-sensitive glycine receptors." J Biol Chem **280**(45): 37877-37884.

Miyazawa, A., Y. Fujiyoshi and N. Unwin (2003). "Structure and gating mechanism of the acetylcholine receptor pore." Nature **423**(6943): 949-955.

Molander, A., H. H. Lido, E. Lof, M. Ericson and B. Soderpalm (2007). "The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male wistar rats." Alcohol Alcohol **42**(1): 11-18.

Molander, A., E. Lof, R. Stomberg, M. Ericson and B. Soderpalm (2005). "Involvement of accumbal glycine receptors in the regulation of voluntary ethanol intake in the rat." Alcohol Clin Exp Res **29**(1): 38-45.

Molander, A. and B. Soderpalm (2005). "Accumbal strychnine-sensitive glycine receptors: an access point for ethanol to the brain reward system." Alcohol Clin Exp Res **29**(1): 27-37.

Mori, M., B. H. Gahwiler and U. Gerber (2002). "Beta-alanine and taurine as endogenous agonists at glycine receptors in rat hippocampus in vitro." J Physiol **539**(Pt 1): 191-200.

Morris, M. C., S. Deshayes, F. Heitz and G. Divita (2008). "Cell-penetrating peptides: from molecular mechanisms to therapeutics." Biol Cell **100**(4): 201-217.

Murail, S., B. Wallner, J. R. Trudell, E. Bertaccini and E. Lindahl (2011). "Microsecond Simulations Indicate that Ethanol Binds between Subunits and Could Stabilize an Open-State Model of a Glycine Receptor." Biophys J **100**(7): 1642-1650.

Narahashi, T., G. L. Aistrup, W. Marszalec and K. Nagata (1999). "Neuronal nicotinic acetylcholine receptors: a new target site of ethanol." Neurochem Int **35**(2): 131-141.

Newland, C. F. and S. G. Cull-Candy (1992). "On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat." J Physiol **447**: 191-213.

Nury, H., C. Van Renterghem, Y. Weng, A. Tran, M. Baaden, V. Dufresne, J. P. Changeux, J. M. Sonner, M. Delarue and P. J. Corringer (2011). "X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel." Nature **469**(7330): 428-431.

Ortells, M. O. and G. G. Lunt (1995). "Evolutionary history of the ligand-gated ion-channel superfamily of receptors." Trends Neurosci **18**(3): 121-127.

Oyama, T., I. T. Rombel, K. N. Samli, X. Zhou and K. C. Brown (2006). "Isolation of multiple cell-binding ligands from different phage displayed-peptide libraries." Biosens Bioelectron **21**(10): 1867-1875.

Pasqualini, R. and E. Ruoslahti (1996). "Tissue targeting with phage peptide libraries." Mol Psychiatry **1**(6): 423.

Peters, E. A., P. J. Schatz, S. S. Johnson and W. J. Dower (1994). "Membrane insertion defects caused by positive charges in the early mature region of protein pIII of filamentous phage fd can be corrected by prlA suppressors." J Bacteriol **176**(14): 4296-4305.

Pfeiffer, F., D. Graham and H. Betz (1982). "Purification by affinity chromatography of the glycine receptor of rat spinal cord." J Biol Chem **257**(16): 9389-9393.

Pratt, D., H. Tzagoloff and W. S. Erdahl (1966). "Conditional lethal mutants of the small filamentous coliphage M13. I. Isolation, complementation, cell killing, time of cistron action." Virology **30**(3): 397-410.

Pribilla, I., T. Takagi, D. Langosch, J. Bormann and H. Betz (1992). "The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels." Embo J **11**(12): 4305-4311.

Pringle, M. J., K. B. Brown and K. W. Miller (1981). "Can the lipid theories of anesthesia account for the cutoff in anesthetic potency in homologous series of alcohols?" Mol Pharmacol **19**(1): 49-55.

Rajendra, S. and P. R. Schofield (1995). "Molecular mechanisms of inherited startle syndromes." Trends Neurosci **18**(2): 80-82.

Rakonjac, J., N. J. Bennett, J. Spagnuolo, D. Gagic and M. Russel (2011). "Filamentous Bacteriophage: Biology, Phage Display and Nanotechnology Applications." Curr Issues Mol Biol **13**(2): 51-76.

Rakonjac, J., J. Feng and P. Model (1999). "Filamentous phage are released from the bacterial membrane by a two-step mechanism involving a short C-terminal fragment of pIII." J Mol Biol **289**(5): 1253-1265.

Rakonjac, J. and P. Model (1998). "Roles of pIII in filamentous phage assembly." J Mol Biol **282**(1): 25-41.

Rapoport, M. and H. Lorberboum-Galski (2009). "TAT-based drug delivery system--new directions in protein delivery for new hopes?" Expert Opin Drug Deliv **6**(5): 453-463.

Rebar, E. J. and C. O. Pabo (1994). "Zinc finger phage: affinity selection of fingers with new DNA-binding specificities." Science **263**(5147): 671-673.

Roberts, M. T., R. Phelan, B. S. Erlichman, R. N. Pillai, L. Ma, G. F. Lopreato and S. J. Mihic (2006). "Occupancy of a single anesthetic binding pocket is sufficient to enhance glycine receptor function." J Biol Chem **281**(6): 3305-3311.

Rodi, D. J., A. S. Soares and L. Makowski (2002). "Quantitative assessment of peptide sequence diversity in M13 combinatorial peptide phage display libraries." J Mol Biol **322**(5): 1039-1052.

Rothlin, C. V., E. Katz, M. Verbitsky and A. B. Elgoyhen (1999). "The alpha9 nicotinic acetylcholine receptor shares pharmacological properties with type A gamma-aminobutyric acid, glycine, and type 3 serotonin receptors." Mol Pharmacol **55**(2): 248-254.

Roux, S., E. Zekri, B. Rousseau, M. Paternostre, J. C. Cintrat and N. Fay (2008). "Elimination and exchange of trifluoroacetate counter-ion from cationic peptides: a critical evaluation of different approaches." J Pept Sci **14**(3): 354-359.

Ruiz-Gomez, A., E. Morato, M. Garcia-Calvo, F. Valdivieso and F. Mayor, Jr. (1990). "Localization of the strychnine binding site on the 48-kilodalton subunit of the glycine receptor." Biochemistry **29**(30): 7033-7040.

Ruiz-Gomez, A., M. L. Vaello, F. Valdivieso and F. Mayor, Jr. (1991). "Phosphorylation of the 48-kDa subunit of the glycine receptor by protein kinase C." J Biol Chem **266**(1): 559-566.

Russel, M. (1991). "Filamentous phage assembly." Mol Microbiol **5**(7): 1607-1613.

Russel, M. (1993). "Protein-protein interactions during filamentous phage assembly." J Mol Biol **231**(3): 689-697.

Russel, M. (1995). "Moving through the membrane with filamentous phages." Trends Microbiol **3**(6): 223-228.

Russel, M. and P. Model (1989). "Genetic analysis of the filamentous bacteriophage packaging signal and of the proteins that interact with it." J Virol **63**(8): 3284-3295.

Saggio, I., I. Gloaguen, G. Poiana and R. Laufer (1995). "CNTF variants with increased biological potency and receptor selectivity define a functional site of receptor interaction." Embo J **14**(13): 3045-3054.

Schwartz, T. W. and B. Holst (2007). "Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act?" Trends Pharmacol Sci **28**(8): 366-373.

Sidhu, S. S., H. B. Lowman, B. C. Cunningham and J. A. Wells (2000). "Phage display for selection of novel binding peptides." Methods Enzymol **328**: 333-363.

Siegel, D. L., T. Y. Chang, S. L. Russell and V. Y. Bunya (1997). "Isolation of cell surface-specific human monoclonal antibodies using phage display and magnetically-activated cell sorting: applications in immunohematology." J Immunol Methods **206**(1-2): 73-85.

Smith, G. P. (1985). "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface." Science **228**(4705): 1315-1317.

Smith, G. P. and V. A. Petrenko (1997). "Phage Display." Chem Rev **97**(2): 391-410.

Soderpalm, B., E. Lof and M. Ericson (2009). "Mechanistic studies of ethanol's interaction with the mesolimbic dopamine reward system." Pharmacopsychiatry **42 Suppl 1**: S87-94.

Song, Y. M. and L. Y. Huang (1990). "Modulation of glycine receptor chloride channels by cAMP-dependent protein kinase in spinal trigeminal neurons." Nature **348**(6298): 242-245.

Stemmer, W. P. (1994). "DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution." Proc Natl Acad Sci U S A **91**(22): 10747-10751.

Szardenings, M., S. Tornroth, F. Mutulis, R. Muceniece, K. Keinanen, A. Kuusinen and J. E. Wikberg (1997). "Phage display selection on whole cells yields a peptide specific for melanocortin receptor 1." J Biol Chem **272**(44): 27943-27948.

Takacs, Z., M. Toups, A. Kollewe, E. Johnson, L. G. Cuello, G. Driessens, M. Biancalana, A. Koide, C. G. Ponte, E. Perozo, T. F. Gajewski, G. Suarez-Kurtz, S. Koide and S. A. Goldstein (2009). "A designer ligand specific for Kv1.3 channels from a scorpion neurotoxin-based library." Proc Natl Acad Sci U S A **106**(52): 22211-22216.

Tamai, I. and A. Tsuji (2000). "Transporter-mediated permeation of drugs across the blood-brain barrier." J Pharm Sci **89**(11): 1371-1388.

Temsamani, J. and P. Vidal (2004). "The use of cell-penetrating peptides for drug delivery." Drug Discov Today **9**(23): 1012-1019.

Tipps, M. E., J. E. Lawshe, A. D. Ellington and S. J. Mihic (2010). "Identification of novel specific allosteric modulators of the glycine receptor using phage display." J Biol Chem **285**(30): 22840-22845.

Treistman, S. N. and G. E. Martin (2009). "BK Channels: mediators and models for alcohol tolerance." Trends Neurosci **32**(12): 629-637.

Trombley, P. Q., L. J. Blakemore and B. J. Hill (2011). "Zinc modulation of glycine receptors." Neuroscience.

Turecek, R. and L. O. Trussell (2001). "Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse." Nature **411**(6837): 587-590.

Unwin, N. (1995). "Acetylcholine receptor channel imaged in the open state." Nature **373**(6509): 37-43.

Vaello, M. L., A. Ruiz-Gomez, J. Lerma and F. Mayor, Jr. (1994). "Modulation of inhibitory glycine receptors by phosphorylation by protein kinase C and cAMP-dependent protein kinase." J Biol Chem **269**(3): 2002-2008.

van den Pol, A. N. and T. Gorcs (1988). "Glycine and glycine receptor immunoreactivity in brain and spinal cord." J Neurosci **8**(2): 472-492.

Vengeliene, V., A. Bilbao, A. Molander and R. Spanagel (2008). "Neuropharmacology of alcohol addiction." Br J Pharmacol **154**(2): 299-315.

Vengeliene, V., F. Leonardi-Essmann, W. H. Sommer, H. M. Marston and R. Spanagel (2010). "Glycine transporter-1 blockade leads to persistently reduced relapse-like alcohol drinking in rats." Biol Psychiatry **68**(8): 704-711.

Vogt, K., J. Mellor, G. Tong and R. Nicoll (2000). "The actions of synaptically released zinc at hippocampal mossy fiber synapses." Neuron **26**(1): 187-196.

Waldvogel, H. J., K. Baer, K. L. Allen, M. I. Rees and R. L. Faull (2007). "Glycine receptors in the striatum, globus pallidus, and substantia nigra of the human brain: an immunohistochemical study." J Comp Neurol **502**(6): 1012-1029.

Wang, D. S., R. Buckinx, H. Lecorronc, J. M. Mangin, J. M. Rigo and P. Legendre (2007). "Mechanisms for picrotoxinin and picrotin blocks of alpha2 homomeric glycine receptors." J Biol Chem **282**(22): 16016-16035.

Weiss, G. A., C. K. Watanabe, A. Zhong, A. Goddard and S. S. Sidhu (2000). "Rapid mapping of protein functional epitopes by combinatorial alanine scanning." Proc Natl Acad Sci U S A **97**(16): 8950-8954.

Wells, J. A. (1991). "Systematic mutational analyses of protein-protein interfaces." Methods Enzymol **202**: 390-411.

Welsh, B. T., B. E. Goldstein and S. J. Mihic (2009). "Single-channel analysis of ethanol enhancement of glycine receptor function." J Pharmacol Exp Ther **330**(1): 198-205.

Werman, R., R. A. Davidoff and M. H. Aprison (1967). "Inhibition of motoneurones by iontophoresis of glycine." Nature **214**(5089): 681-683.

White, J. M. (1992). "Membrane fusion." Science **258**(5084): 917-924.

Wilkemeyer, M. F., S. Y. Chen, C. E. Menkari, D. E. Brenneman, K. K. Sulik and M. E. Charness (2003). "Differential effects of ethanol antagonism and neuroprotection in peptide fragment NAPVSIPQ prevention of ethanol-induced developmental toxicity." Proc Natl Acad Sci U S A **100**(14): 8543-8548.

Wilkemeyer, M. F., C. E. Menkari, C. Y. Spong and M. E. Charness (2002). "Peptide antagonists of ethanol inhibition of l1-mediated cell-cell adhesion." J Pharmacol Exp Ther **303**(1): 110-116.

Williams, K. L., A. P. Ferko, E. J. Barbieri and G. J. DiGregorio (1995). "Glycine enhances the central depressant properties of ethanol in mice." Pharmacol Biochem Behav **50**(2): 199-205.

Wrighton, N. C., F. X. Farrell, R. Chang, A. K. Kashyap, F. P. Barbone, L. S. Mulcahy, D. L. Johnson, R. W. Barrett, L. K. Jolliffe and W. J. Dower (1996). "Small peptides as potent mimetics of the protein hormone erythropoietin." Science **273**(5274): 458-464.

Yamane, K. and S. Mizushima (1988). "Introduction of basic amino acid residues after the signal peptide inhibits protein translocation across the cytoplasmic membrane of Escherichia coli. Relation to the orientation of membrane proteins." J Biol Chem **263**(36): 19690-19696.

Yang, Z., B. A. Cromer, R. J. Harvey, M. W. Parker and J. W. Lynch (2007). "A proposed structural basis for picrotoxinin and picrotin binding in the glycine receptor pore." J Neurochem **103**(2): 580-589.

Ye, J. H., K. A. Sokol and U. Bhavsar (2009). "Glycine receptors contribute to hypnosis induced by ethanol." Alcohol Clin Exp Res **33**(6): 1069-1074.

Ye, J. H., L. Tao, J. Ren, R. Schaefer, K. Krnjevic, P. L. Liu, D. A. Schiller and J. J. McArdle (2001a). "Ethanol potentiation of glycine-induced responses in dissociated neurons of rat ventral tegmental area." J Pharmacol Exp Ther **296**(1): 77-83.

Ye, J. H., L. Tao, L. Zhu, K. Krnjevic and J. J. McArdle (2001b). "Ethanol inhibition of glycine-activated responses in neurons of ventral tegmental area of neonatal rats." J Neurophysiol **86**(5): 2426-2434.

Ye, J. H., F. Wang, K. Krnjevic, W. Wang, Z. G. Xiong and J. Zhang (2004). "Presynaptic glycine receptors on GABAergic terminals facilitate discharge of dopaminergic neurons in ventral tegmental area." J Neurosci **24**(41): 8961-8974.

Ye, Q., V. V. Koltchine, S. J. Mihic, M. P. Mascia, M. J. Wick, S. E. Finn, N. L. Harrison and R. A. Harris (1998). "Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position alpha267." J Biol Chem **273**(6): 3314-3319.

Yevenes, G. E., G. Moraga-Cid, A. Avila, L. Guzman, M. Figueroa, R. W. Peoples and L. G. Aguayo (2010). "Molecular requirements for ethanol differential allosteric modulation of glycine receptors based on selective Gbetagamma modulation." J Biol Chem **285**(39): 30203-30213.

Yevenes, G. E., G. Moraga-Cid, R. W. Peoples, G. Schmalzing and L. G. Aguayo (2008). "A selective G betagamma-linked intracellular mechanism for modulation of a ligand-gated ion channel by ethanol." Proc Natl Acad Sci U S A **105**(51): 20523-20528.

Yevenes, G. E., R. W. Peoples, J. C. Tapia, J. Parodi, X. Soto, J. Olate and L. G. Aguayo (2003). "Modulation of glycine-activated ion channel function by G-protein betagamma subunits." Nat Neurosci **6**(8): 819-824.

Young, A. B. and S. H. Snyder (1973). "Strychnine binding associated with glycine receptors of the central nervous system." Proc Natl Acad Sci U S A **70**(10): 2832-2836.

Zacharias, N. and D. A. Dougherty (2002). "Cation-pi interactions in ligand recognition and catalysis." Trends Pharmacol Sci **23**(6): 281-287.

Zacher, A. N., 3rd, C. A. Stock, J. W. Golden, 2nd and G. P. Smith (1980). "A new filamentous phage cloning vector: fd-tet." Gene **9**(1-2): 127-140.

Zaman, G. J., A. M. Kaan, J. G. Schoenmakers and R. N. Konings (1992). "Gene V protein-mediated translational regulation of the synthesis of gene II protein of the filamentous bacteriophage M13: a dispensable function of the filamentous-phage genome." J Bacteriol **174**(2): 595-600.

Zarbin, M. A., J. K. Wamsley and M. J. Kuhar (1981). "Glycine receptor: light microscopic autoradiographic localization with [3H]strychnine." J Neurosci **1**(5): 532-547.

Zeilhofer, H. U. (2005). "The glycinergic control of spinal pain processing." Cell Mol Life Sci **62**(18): 2027-2035.

Zenkin, N., T. Naryshkina, K. Kuznedelov and K. Severinov (2006). "The mechanism of DNA replication primer synthesis by RNA polymerase." Nature **439**(7076): 617-620.

Zhang, Y., M. J. Laster, K. Hara, R. A. Harris, E. I. Eger, 2nd, C. R. Stabernack and J. M. Sonner (2003). "Glycine receptors mediate part of the immobility produced by inhaled anesthetics." Anesth Analg **96**(1): 97-101, table of contents.

Zhu, L. and J. H. Ye (2005). "The role of G proteins in the activity and ethanol modulation of glycine-induced currents in rat neurons freshly isolated from the ventral tegmental area." Brain Res **1033**(1): 102-108.

Zoller, M. J. and M. Smith (1982). "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA." Nucleic Acids Res **10**(20): 6487-6500.

Zoller, M. J. and M. Smith (1983). "Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors." Methods Enzymol **100**: 468-500.

Vita

Megan Elizabeth Tipps was born in Amarillo, Texas to David and Elizabeth Tipps. She has two younger sisters, Hillary and Kelsey Tipps, and was raised in Muleshoe, Texas. Megan attended Baylor University in Waco, Texas, where she received a Bachelor of Science degree in Neuroscience in December of 2005. In the Fall of 2006, she was admitted to the graduate program of the Institute for Neuroscience at the University of Texas at Austin.

Permanent address: 7401 SW Aloma Wy. #3, Portland, OR 97223

metipps@gmail.com

This dissertation was typed by the author.